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NMR Metabolomics Analysis of Parkinson's Disease

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Abstract: Parkinson's disease (PD) is a neurodegenerative disease, which is characterized by progressive death of dopaminergic neurons in the substantia nigra pars compacta. Although mitochondrial dysfunction and oxidative stress are linked to PD pathogenesis, its etiology and pathology remain to be elucidated. Metabolomics investigates metabolite changes in biofluids, cell lysates, tissues and tumors in order to correlate these metabolomic changes to a disease state. Thus, the application of metabolomics to investigate PD provides a systematic approach to understand the pathology of PD, to identify disease biomarkers, and to complement genomics, transcriptomics and proteomics studies. This review will examine current research into PD mechanisms with a focus on mitochondrial dysfunction and oxidative stress. Neurotoxin-based PD animal models and the rationale for metabolomics studies in PD will also be discussed. The review will also explore the potential of NMR metabolomics to address important issues related to PD treatment and diagnosis.

Keywords: Biomarkers, metabolomics, NMR, OPLS, Parkinson's disease, PCA, PLS.

1. INTRODUCTION

PD is the second most common neurodegenerative disorder behind Alzheimer's disease [1-3]. In 2005, 4.1 to 4.6 million people were estimated to have PD worldwide, with a high prevalence of PD in the United States [4]. Aging is one of the strongest risk factors associated with PD (Fig. **1a**) [5, 6]. Correspondingly, as the world population continues to age, the number of PD cases is predicted to double by 2030, imposing an increasing burden on the healthcare systems in many countries. In the U.S.A. alone, the annual economic cost of Parkinson's disease is estimated at $10.8 billion [7]. As a result, maintenance of functional independence at advanced ages has become a critical public health priority [8].

PD patients suffer from a range of movement disorders (bradykinesia, postural instability, rigidity, tremors) collectively known as Parkinsonism. Parkinsonism is mainly caused by the degeneration of dopaminergic neurons (DAergic) in the substantia nigra pars compacta (SNpc) and the resulting depletion in dopamine. However, a large proportion of PD patients also suffer from other non-motor symptoms (anosmia, autonomic dysfunction, hallucinations, sleep disorders) [10]; along with neuronal loss in many other brain regions. This can occur before or after the loss of DAergic neurons [11, 12]. Currently, the diagnosis of PD depends primarily on the observation of motor symptoms in a clinical setting. Unfortunately, the rate of misdiagnosis of PD may be upwards of 50% [13]. This is due to the absence of symptoms in the early stages of the disease, and a variation of symptoms among individual patients [14]. There is also no cure for PD. Current treatments focus on addressing the symptoms and reducing the progression of the disease [15-18]. The standard treatment for PD consists of replacing dopamine with L-DOPA (Fig. **2**), a dopamine precursor [19]. Unfortunately, the effectiveness of L-DOPA declines as the disease progresses; leading to serious side-effects.

Despite decades of investigation, the PD mechanism of pathogenesis is still unknown and dopamine depletion may be merely a consequence of PD. Several studies have shown that proteinaceous inclusions (Lewy bodies and Lewy neuritis) are localized in different areas of a PD brain (Fig. **1b**) even before dopamine loss and Parkinsonism symptoms occur [20-22]. These proteinaceous inclusions are composed of lipids and several proteins, such as α-synuclein (PARK1) [23]. Correspondingly, current hypotheses for PD pathogenesis are based on protein misfolding and aggregation [24], mitochondrial dysfunction [25], or oxidative stress [26]. Initially, these pathogenesis routes were thought to work independently, but recent data strongly suggests they interact together to deplete dopamine [27].

In this review, we will briefly summarize current models and supportive evidence for the molecular mechanisms of PD, with a specific focus on mitochondrial dysfunction and oxidative stress. We will provide a background and rationale for the utility of metabolomics to investigate PD with a particular emphasis on the application of NMR-based metabolomics. Metabolomics has a tremendous potential to provide valuable insights into the etiopathogenesis of PD, to discover novel molecular targets for the treatment of PD, and to identify reliable and sensitive PD biomarkers. Biomarkers may play an important role in the early diagnosis of PD, in monitoring the progression of PD, and in determining the efficacy of therapeutic intervention. Metabolomics methodology and its application to PD will also be briefly described. The proper choice and handling of cellular and animal models are critical to a successful metabolomics study. Thus, the most commonly used animal PD models and their application to metabolomics will also be summarized.

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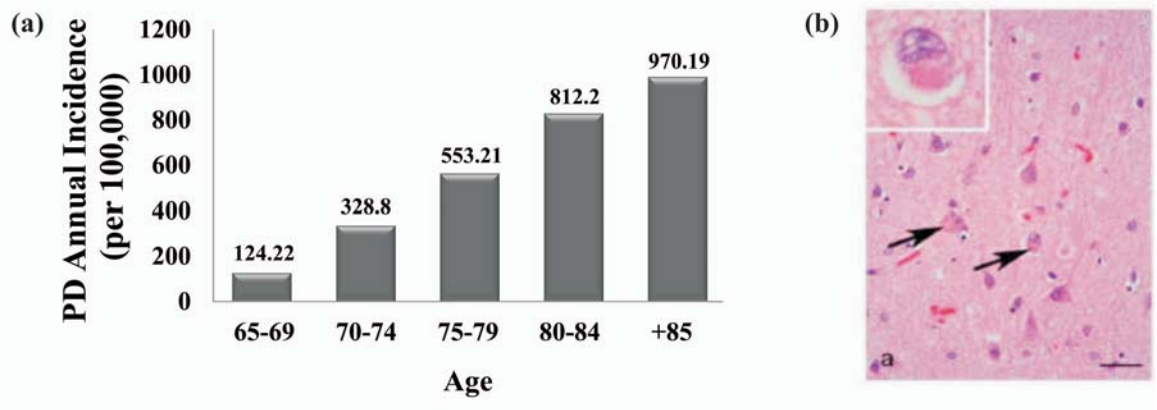

Fig. (1). (a) Annual US incidence of Parkinson's disease as a function of the patient's age. The occurrence of PD below age 65 is rare [6]. **(b)** α-synuclein aggregates in the cortex of patients with Lewy body dementia can be shown in the shape of Lewy bodies by using conventional histological techniques; Bar=50 μm (HE, arrow, inset) (Reprinted with permission from reference [9], Copyright 2010 by Deutscher Ärzte-Verlag GmbH).

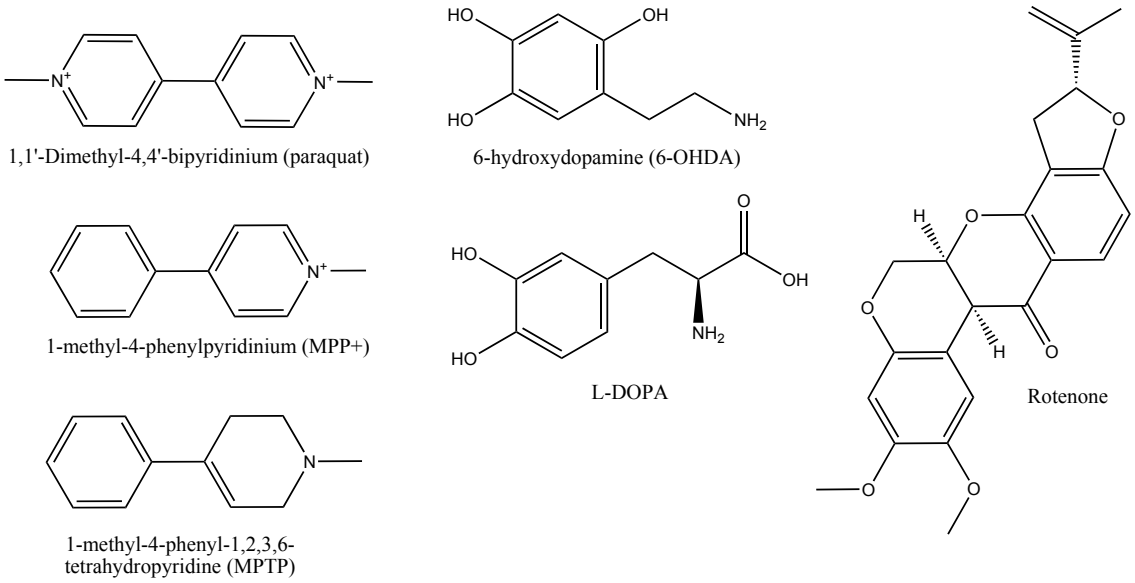

Fig. (2). Chemical structures of L-DOPA, a common treatment for Parkinson's disease, and toxins, herbicides and pesticides known to selectively destroy dopamine neurons and induce Parkinson's disease in animals.

2. DISCUSSION

2.1. Risk Factors of PD

Studies on the etiology of PD have revealed multiple factors. In addition to aging, PD has been shown to have a genetic association [28], and a correlation with head trauma [29] and exposure to pesticides and herbicides (Fig. **2**) [30]. Specifically, PD has been associated with exposure to paraquat, carbamates, and organochlorines [31, 32]. Not surprisingly, the Midwest of the USA have a high prevalence of PD with Nebraska having the highest number of PD patients per capita [6]. A byproduct of a synthetic opioid (MPPP, 1-methyl-4-phenyl-4-propionoxypiperidine), 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is chemically similar to paraquat and also results in PD-like motor symptoms and further supports an environmental cause of PD.

Additionally, there are genetic links that associate PD with pesticides and herbicides [33]. In fact, a recent study suggests that an environmental-genetic interaction plays a more important role in PD than do either genetic or environmental factors alone [34]. A genetic analysis of an Italian family [35] and a western Nebraska family [36] identified an association between the α-synuclein (PARK1) and leucine-rich repeat kinase 2 (LRRK2/PARK8) genes and the development of both familial and sporadic PD. Similar genetic studies have identified mutations in the protein Parkin (PARK2) as the most common indicator of familial PD [37]. Other genetic studies have identified a correlation with PD for mutations in lysosomal protein glucocerebrosidase (GBA) [38], PTEN-induced putative kinase 1 (PINK1/PARK6) [39], and the DJ-1 protein (PARK7) [40]. The majority of PD cases are sporadic [1], with weak genetic contributions. In the remaining, familial cases of PD, causal genes could be identified. α-synuclein (PARK1) and leucine-rich repeat kinase 2 (LRRK2/PARK8) are associated with autosomal-dominant forms of PD and Parkin (PARK2), PTEN-induced putative kinase 1 (PINK1/PARK6), DJ-1 protein (PARK7) are associated with autosomal recessive forms of PD [41].

Mutations in leucine-rich repeat kinase 2 (LRRK2/PARK8) usually results in mid-to-late onset PD with a slow disease progression through an unknown mechanism. Correspondingly, the neuropathology is generally inconsistent. Conversely, mutations in α-synuclein (PARK1) often lead to early-onset PD with a rapid disease progression and the presence of Lewy bodies and α-synuclein (PARK1) fibrils. The mutation in GBA possibly leads to an increase in α-synuclein (PARK1) aggregation [42]. Parkin (PARK2) mutations results in juvenile and very early-onset PD that progresses very slowly. It causes neuronal loss and gliosis in the substantia nigra, but commonly lacks Lewy bodies. Indistinguishable clinical outcomes occur with mutations in PINK1 (PARK6) and DJ-1/PARK7. Importantly, mutations in Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7) and exposure to pesticides and herbicides appear to alter redox balance leading to neuronal death [43]. Parkin (PARK2) is involved in protein ubiquitination and interacts with synphilin-1, which interacts with α-synuclein (PARK1). DJ-1/PARK7 has an unknown function, but has been associated with a protective role related to oxidative stress. PINK1 (PARK6) is upstream of Parkin (PARK2) in a pathway associated with eliminating damaged mitochondria [39, 44, 45]. The mitochondria are a major provider of cellular energy and also play a key role in cell death (apoptosis) [46, 47]. The brain has the highest energy requirement of all organs and, correspondingly, neuronal mitochondria are subjected to high levels of oxidative stress and potential damage that may lead to cell death. As a result, mitochondrial quality control pathways have evolved to maintain molecular function or eliminate a dysfunctional organelle, where the impairment of these processes is associated with PD [44, 45].

2.2. Mitochondrial Dysfunction and Oxidative Stress in PD

Oxidative stress is caused by a cumulative production of free radicals, typically known as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Elevated level of ROS may result in a significant damage to DNA, proteins and lipids [48]. Brain tissues are more susceptible to oxidative stress due to (i) a low concentration of antioxidant enzymes and a low ability to maintain energy homeostasis [49], (ii) a high consumption of the total available oxygen [50, 51], (iii) a high percentage of polyunsaturated fatty acids [52], and (iv) the increased presence of redox metals (iron, copper and zinc) in an aging brain [53]. Among many sources of free radical production, metal-catalyzed reactions and mitochondria-catalyzed electron transport reactions are two potential sources of PD-related ROS [54]. It is thought that 95-98% of ROS generated during aerobic metabolism are from mitochondrial activity [55]. As a result, mitochondria contain an extensive antioxidant defense system, where a damaged or dysfunctional mitochondrion leads to an increase in ROS [56].

Dopaminergic neurons in the substantia nigra pars compacta region are also very prone to oxidative damage because of (i) higher iron and copper levels in this region of the brain, higher activity of monoamine oxidase, and aberrant oxidation of dopamine that all lead to a higher intrinsic ROS, (ii) higher sensitivity to ROS signaling that induces apoptosis, (iii) lower mitochondrial mass, lower ATP levels and dysfunctional mitochondria, (iv) readily activated mitochondrial permeability transition (MPT) pore by dopamine metabolite that leads to cell death, (v) deficient DNA damage repair, (vi) glial dysfunction, (vii) calcium dysregulation and glutamate hyperactivity, and (viii) the large size of the neurons and higher demand for energy [57, 58].

Mitochondria were implicated in PD by two cases involving young drug users taking illicit narcotics contaminated with MPTP [59]. Later studies implicated the MPP+ (1-methyl-4-phenylpyridinium) metabolite of MPTP (Fig. **2**) as the cause of Parkinsonism in these designer-drug abusers. MPP+ inhibits complex I of the mitochondrial electron-transport chain [60]. This model has been supported by animal studies, in which the chronic infusion of rotenone [61] (another complex-I inhibitor) or MPTP [62] results in a clinical parkinsonian phenotype, and pathological nigrostriatal dopamine degeneration with cytoplasmic inclusions immunoreactive for α-synuclein (PARK1) and ubiquitin [63]. Oxidative stress is the probable mechanism of toxicity for complex-I inhibitors [64]. Complex-I inhibition and oxidative stress were shown to be relevant to naturally occurring PD when complex-I deficiency and glutathione depletion were found in the substantia nigra of patients with idiopathic PD, and in patients with pre-symptomatic PD [65]. In addition, many of the genes associated with PD also have a functional role in the mitochondria. Choi *et al.* argues that mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by retenone, MPP+, or paraquat. A mouse strain lacking functional Ndufs4, which abolishes complex I activity in midbrain mesencephalic neurons cultured from embryonic day 14 mice, showed no effect on the survival of dopaminergic neurons in culture [66].

2.3. PD Animal Models

Animal models are essential tools to study etiology, pathogenesis, and the molecular mechanisms of PD *in vivo*. Over the past decades, various animal models have been developed for PD research [67-69]. These animal models can be generally categorized into toxin-based models and genetic models.

Toxin-based models typically rely on dosing animals with MPTP, 6-hydroxydopamine (6-OHDA), rotenone or paraquat. Given the well-known cases of PD-like motor symptoms induced by MPTP associated drugs, MPTP has been widely used as a model for PD. Administration of MPTP mimics human PD in many aspects, such as selective lesion of substrantia nigra dopaminergic neurons and the presence of α-synuclein (PARK1) aggregates. Unlike PD, the MPTP animal model is an acute, non-progressive disease. The neuron toxin 6-OHDA has the longest history of use as a PD model and has been commonly used to degenerate central catecholaminergic projections, including the nigrostriatal system [70, 71]. Although dopamine depletion, nigral dopamine cell loss, and neurobehavioral deficits have been observed with the 6-OHDA model, it does not mimic all of the clinical features of PD. The 6-OHDA animal model does not form Lewy bodies, and it does not affect other PD involved brain regions, such as olfactory structures, lower brain stem areas, or locus coeruleus [72]. The rotenone animal model is the first to link an environmental toxin to PD

development [73, 74]. Rotenone administration produces hallmark traits of PD, such as nigrostriatal dopamine neurons damage, α-synuclein (PARK1) aggregation, Lewy-like body formation, oxidative stress, and gastrointestinal problems [75].

A number of genetic animal models have been created by mutating PD associated genes, such as, α-synuclein (PARK1) [76, 77], LRRK2 (PARK8) [78, 79], PINK1 (PARK6), Parkin (PARK2) [80, 81], DJ-1 (PARK7) [82] and ubiquitin C-terminal hydrolase-L1 (PARK5) [83]. Interestingly, these genetic models do not present PD related neuronal degeneration, but they provide systems for investigating PD pathogenesis, for evaluating potential therapeutic targets, and to assist in the drug discovery process.

2.4. The Rationale for PD Metabolomics Studies

Metabolomics refers to the study of the metabolome, the collection of small molecules, such as amino acids, carbohydrates and lipids, present in cells, tissues, organs, or biological fluids [84-90]. Metabolomics combines analytical techniques (IR, NMR, MS, etc.) [86, 91, 92] with multivariate statistical methods [93] to analyze metabolite concentration changes in a high throughput manner. Metabolomics is a relatively new omics discipline that is complementary to genomics and proteomics, and an important addition to systems biology [87, 94-96]. The identities, concentrations, and fluxes of the metabolites within a metabolome are a direct consequence of protein activity, and, importantly, change in response to the environment (disease state, drug treatment, nutrient availability, genetic modification, etc.). Thus, the metabolome reflects the state of a cell or biological system and can provide an overall picture of how the system responds to a specific perturbation. Thus, metabolomics is routinely used to define phenotypes [97-99]. Conversely, a change in the expression level of a gene or protein from a genomics or proteomics study is not necessarily correlated with a change in protein activity, or directly linked to a disease state. In addition to studying disease pathogenesis, metabolomics is used in drug discovery to identify chemical leads [100] and novel therapeutic targets [101]. Metabolomics is also a powerful tool for the identification of biomarkers for early disease detection, for monitoring disease progression, and the response to therapy [84]. Biomarkers have been identified from a variety of bodily fluids that include cerebrospinal fluid (CSF) by NMR imaging and GC-MS [102], feces by NMR [103], saliva by CE-MS [104], serum by NMR [105], and urine by NMR [106]. Metabolomics has been applied to a wide-variety of human diseases including cardiovascular diseases [107], diabetes [108], and various types of cancer [109]. Metabolomics has also been successfully applied to investigate central nervous system disorders such as Alzheimer's Disease [110], Huntington's disease [111], motor neuron disease [112], and schizophrenia [113]. Paige *et al.* reported an alteration in several metabolites between depressed patients and controls by performing an MS-based metabolomics analysis of blood plasma samples [114]. Given that PD pathology may be closely related to protein misfolding and aggregation, mitochondrial dysfunction and oxidative stress, metabolomic changes would also be expected for PD.

PD is a complex and heterogeneous disease where metabolomics holds the promise of identifying specific disease-related networks to enhance our understanding of the multiple interrelated pathways of pathogenesis. This, in turn, may lead to the identification of novel therapeutic targets and the development of new treatments. Moreover, the application of metabolomics may result in the discovery of PD biomarkers for diagnosing the disease. Validated PD biomarkers would aid in the early detection of the disease and address the high misdiagnosis rate for PD [115, 116]. PD biomarkers would also allow for the accurate evaluation of disease progression, which would be invaluable in assessing the efficacy of a patient's treatment [117].

2.5. NMR-based Metabolomics Methodology

Due to the large size and diversity in the chemical and physical properties of the metabolome [118, 119], a range of analytical techniques have been applied to characterize metabolites present in a biological sample. Common analytical techniques used for metabolomics include NMR [86], GC-MS [120], LC-MS [121] and LC-electrochemistry array metabolomics platforms (LCECA) [122]. These analytical techniques have both advantages and limitations; and as a result are complementary to each other. Thus, it is common to integrate multiple techniques in a metabolomics study [123-125]. NMR spectroscopy has been used to investigate a wide range of diseases such as cancers [126-128], aging [129], heart disease [130], polycystic ovary syndrome [131, 132], and diabetes [133]. Correspondingly, NMR has some unique advantages that include: minimal sample handling, high reproducibility, easy quantitation, non-destructive, structural determination, and high throughput [88, 134-137]. Although we only discuss NMR-based methodologies in this review, there is a common work flow that is independent of the analytical method employed. This work flow is comprised of sample collection, sample preparation, data processing and analysis, and metabolite pathway identification (Fig. **3**). However, the experimental details for these processes (sample preparation, data analysis, etc.) still need to be optimized for the specific analytical technique utilized.

Various types of biological samples can be analyzed by NMR which includes cell extracts, tissue extracts, and numerous biofluids. Moreover, intact tissues can be analyzed by using high resolution magic angle spinning magnetic resonance spectroscopy (HR MAS) after a simple sample preparation procedure [138]. For a typical PD *in vitro* study, intracellular metabolites are acquired by quenching and lysing neuron cells, followed by extracting the metabolome. Cerebrospinal fluid (CSF) is a preferred choice for a PD *in vivo* study. However, there are associated risks with obtaining CSF from patients that diminishes its value as a routine diagnostic tool [139, 140]. Instead, easily accessible biofluids (plasma, serum, urine) provide a safer alternative. Procedures for preparing metabolomic samples from mammalian cell lines and biofluids have been extensively reviewed [89, 134, 141-165].

The metabolome is a complex heterogeneous collection of compounds with a diversity of physiochemical properties, where specific metabolite stability and enzymatic turn-over rates are highly variable [162, 163]. It is essential that the

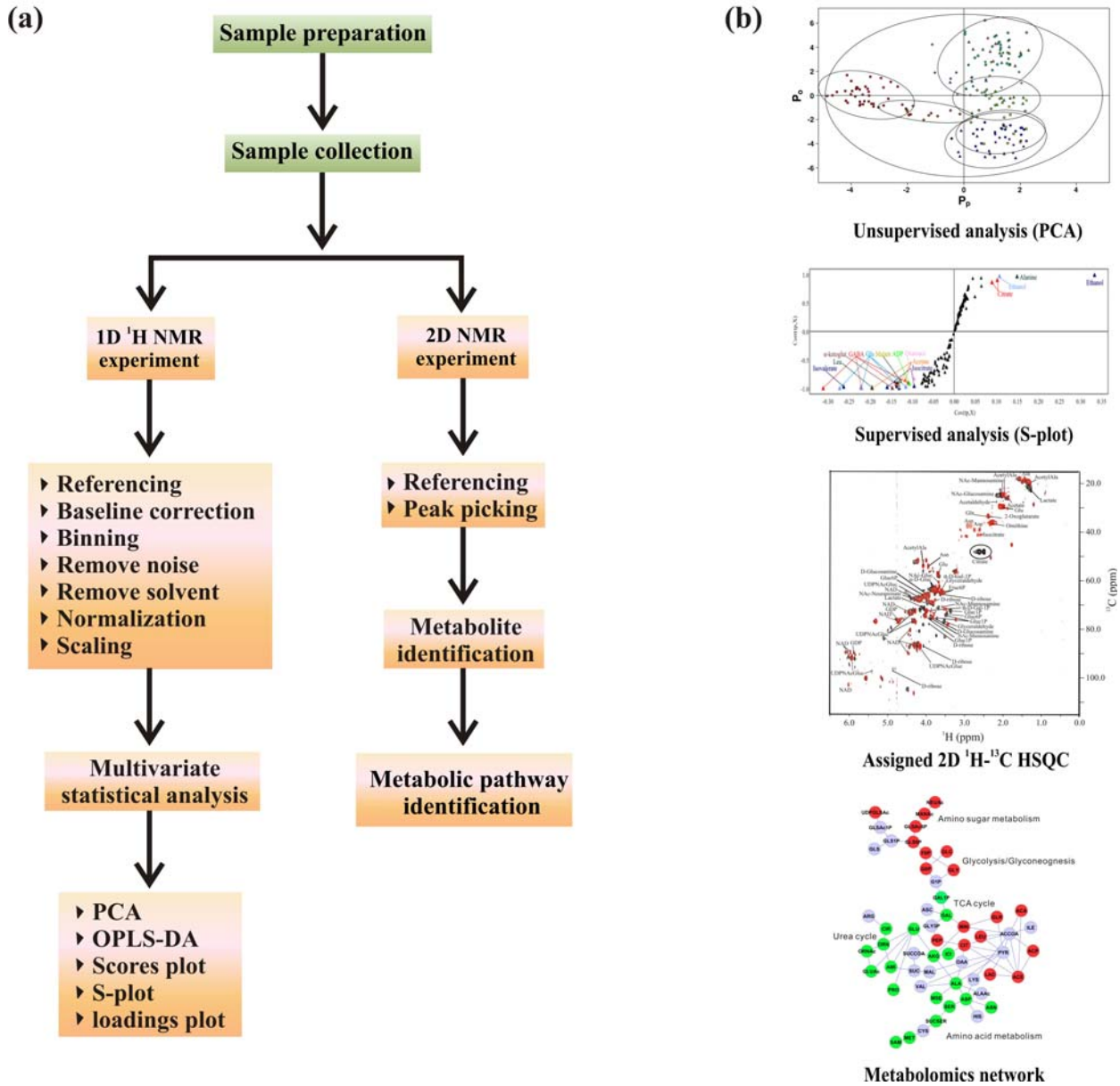

Fig. (3). (a) Illustration of NMR-based metabolomics work flow with **(b)** select examples of representative data and results [100, 159-161].

extracted or collected metabolome reflects the true state of the system and is biologically relevant. In effect, changes to the metabolome should not be introduced by sample handling. Thus, a reliable sampling protocol is critical to a successful metabolomics study, where rapid sample collection and analysis are important [164]. For example, rapid quenching of cells with cold methanol avoids the more time consuming and physiological stressful trypsinization applied in conventional sampling methods [165]. Residual enzymatic activity or induction of stress response pathways (*e.g.* apoptosis) from trypsinization would alter the metabolome. Sample preparation is considerably simpler for biofluids. The sample is filtered or centrifuged; and then spiked with a deuterated phosphate buffer containing a preservative or anticoagulant [89, 90].

The application of NMR for the analysis of metabolomics samples has also been extensively reviewed [84-86, 88, 135, 166-171]. A typical one-dimensional (1D) ^{1}H NMR spectrum can be acquired in a few minutes using an automated high throughput protocol [172]. The resulting 1D ^{1}H NMR spectrum provides a global "fingerprint" or "snapshot" of the metabolome for each sample. Thus, the 1D ^{1}H NMR spectrum is routinely used to characterize a particular class (healthy or diseased, control or drug treated, etc.) and to identify the global features that distinguish between the classes or groups. This initial global analysis of the metabolome doesn't rely on identifying changes to individual metabolites (or assigning the highly complex NMR spectrum). Instead, multivariate statistical methods (principal component analysis, PCA; partial least squares, PLS; orthogonal projection to latent structures, OPLS) are applied to identify the spectral features that distinguish between the classes or groups [93, 173-176]. In general, the outcome of PCA, PLS, or OPLS is a scores plot, where each 1D ^{1}H

NMR spectrum is reduced to a single point in the plot (Fig. **4**). Simply, the NMR spectrum consisting of a range of chemical shifts (ppm) and peak intensities is transformed into a multidimensional Cartesian space, where each axis (V_1, V_2, V_3, … V_n) corresponds to a chemical shift and the value along each axis is the intensity of the NMR peak at that given chemical shift. If the NMR spectrum was collected with 32K data points there would be 32K axes. PCA then identifies a principal component vector ($\overrightarrow{PC_1}$) within this multidimensional space corresponding to the largest variation in the data set. A second vector ($\overrightarrow{PC_2}$) orthogonal to $\overrightarrow{PC_1}$ is identified that corresponds to the next largest variation in the data set. Each successive vector describes a diminishing amount of the variability of the data set, where most of the variability is described by the first two or three principal components. The PC_1 and PC_2 scores (unitless values) are the individual fit of each NMR spectrum to $\overrightarrow{PC_1}$ and $\overrightarrow{PC_2}$, respectively, and are usually presented in a 2D plot. The relative clustering of the NMR spectra in the scores plot identifies the relative similarity or differences between each spectrum, and correspondingly, their metabolomes. In essence, PCA, PLS, or OPLS is used to determine if a statistically significant difference in the metabolome has occurred as a result of the applied environmental stress (disease state, drug treatment, genetic modification, etc.). To be clear, PCA, PLS, and OPLS do not provide a direct analysis of the statistical significance of group separation in a scores plot. Instead, other utilities, such as a Mahalanobis distance metric and T^2 and F distributions are required to return p values for quantitation of PCA, PLS, or OPLS group separations [177].

Multivariate statistical methods are classified into supervised (PLS, OPLS) and unsupervised (PCA) approaches. PCA [173, 174] is the most popular unsupervised algorithm routinely used in metabolomics. Importantly, PCA provides an unbiased view of the clustering patterns for all the conditions under investigation, and should be routinely employed to verify that a spectral difference actually exists. PCA directly analyzes the NMR spectra without any user intervention. If a true difference exists between the various classes, then all members within a class will cluster together and separate from the other classes in the resulting PCA scores plot. This occurs without PCA having any prior knowledge of the class membership for each NMR spectrum. In principal, PCA can actually be used to identify the class membership for each NMR spectrum. Thus, PCA can also be used as a quality control to identify outliers [178]. Of course, this assumes that the samples and spectra were not biased by experimental procedures or data processing.

PLS and OPLS are commonly used supervised methods, [175, 176]. The major difference between PCA and PLS/OPLS is that class membership is defined prior to PLS or OPLS. Thus, OPLS and PLS are inherently biased techniques since class designations are explicitly incorporated into the analysis. In effect, PLS/OPLS will only identify spectral differences that correlate with the manually defined class designations. This occurs regardless of the significance of these differences compared to other spectral features. As a result, an OPLS or PLS scores plot will always show a separation based on the manually defined class designation, even for completely random data [179]. Thus, OPLS and PLS can easily be misleading and requires validation of the model.

Cross validation and permutation testing [180] and CV-ANOVA [181] are routinely employed to validate OPLS and PLS models. For cross validation, the PLS/OPLS models are generated with a subset of the data, where the quality of the fit of the held-out data is measured against the model. The process is repeated numerous times, where different subsets of the data are held-out. The resulting quality assessment (Q^2) statistic has no standard of comparison besides its theoretical maximum of 1 or an empirically acceptable value of ≥ 0.4 [180]. Unfortunately, a large Q^2 value is still possible for an invalid model. The R^2 values provide a measure of the fit of the data to the model, while it is not a measure of cross-validation an $R^2 >> Q^2$ indicates a possible over-fitting of the model. For permutation, the class designations are randomly changed and the quality of the model (Q^2) is assessed and compared against the model with the correct classification. Again, the process is repeated numerous times where the correct classification is expected to yield the largest Q^2 value. Also, a p value can be calculated based on the distribution of Q^2 values for the incorrect class designations relative to the Q^2 value for the correct model. Similarly, CV-ANOVA provides a p value for the PLS/OPLS model based on the cross-validated predictive residuals of a model as a basis for hypothesis testing.

Supervised analyses does provide valuable clues on biomarkers - the metabolites that experienced the largest change and primarily contribute to the class distinction in the PLS or OPLS scores plot. These potential biomarkers

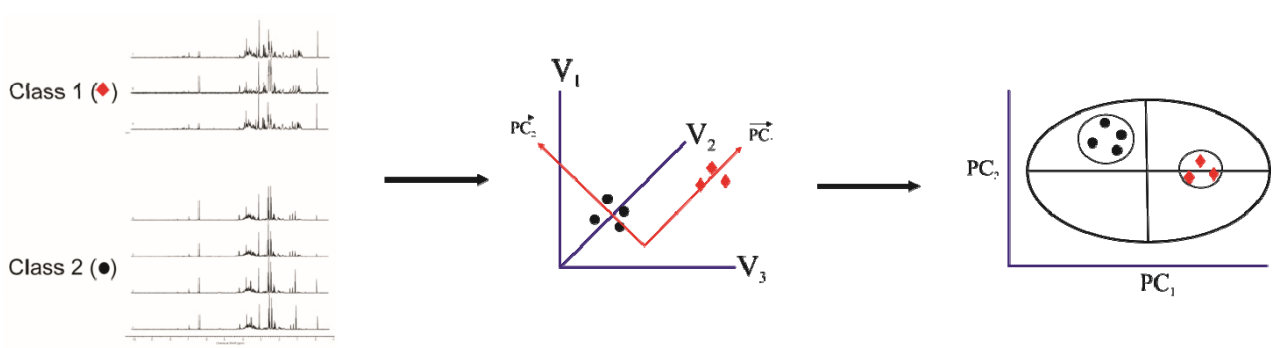

Fig. (4). Illustration of the principal component analysis of NMR spectra.

can then be targeted for further investigation. Potential biomarkers are typically identified from an S-plot [182] generated by the PLS or OPLS model (Fig. **3**). Basically, the extreme regions of the S-plot identify the spectral features (chemical shifts, metabolites) that significantly contribute to the class distinctions observed in the scores plot. A number of other data analysis methods are also used to analyze NMR metabolomics spectra [183], a few select examples include self-organizing map (SOM) [184, 185] support vector machines (SVM) [186, 187], and hierarchical clustering (HCA) [188].

The primary difference between PLS and OPLS is that OPLS attempts to maximize the separation between the classes along the X-axis with unrelated (orthogonal) variations along the Y-axis. As a result, OPLS minimizes within class variations. To be clear, any separation along the Y-axis in an OPLS scores plot is not correlated with the manually defined class designations. In other words, for OPLS a corresponding PLS scores plot is rotated to align the maximal separation between the classes along the X-axis.

Comparable to sample preparation, multivariate statistical methods are also very sensitive to the quality of the input data, where the results can be biased by data handling and processing protocols [179]. Basically, PCA, OPLS or PLS will highlight any spectral difference regardless of the source. Baseline distortions, incorrect phasing, chemical shift referencing errors, chemical shift and line shape perturbations, and irrelevant variations in spectral intensities due to instrument performance or sample preparations are all common NMR issues. NMR chemical shifts are very sensitive to subtle changes in temperature, pH, ionic strength, and instrument stability. Therefore, it is important to implement a uniform data pre-processing protocol that includes spectral alignment, binning, data normalization and data scaling [93]. The spectra can be aligned by using an internal standard like TMSP-d4 or computationally aligned using a variety of methods [189-194]. Importantly, an internal standard can only correct for a uniform deviation in chemical shifts that may arise between replicate samples. For example, chemical shifts generally change linearly with temperature, but a subtle variation in pH may result in large chemical shifts for some peaks, while others are essentially unchanged. Also, the direction of chemical shift change due to pH differences may vary between peaks. NMR spectra are also commonly binned to minimize minor variations in peak position and peak shape, and to filter out noise. The NMR spectrum is divided into "bins" having typical widths of 0.04 ppm, where the total peak intensity within each bin is integrated. "Intelligent" or "adaptive" binning uses variable bin sizes to avoid dividing peaks between multiple bins [195-198]. Since noise is biologically irrelevant and has been shown to bias PCA and PLS, noise regions should be removed prior to any multivariate statistical analysis [199, 200]. In addition to these spectral variations, the total signal intensity may vary across a set of NMR spectra due to differences in the number of cells, biofluid volume, or tissue size per sample. Thus, the absolute NMR peak intensities need to be normalized to a common reference or standard. Common normalization techniques include normalization to the total signal intensity, probabilistic quotient normalization, contrast normalization and quantile normalization [201]. Normalization may also rely on an internal TMSP-d4 standard or an external standard such as cell culture optical density or protein content [202, 203]. Finally, the data needs to be scaled in order to avoid the multivariate analysis from only focusing on changes to the most intense peaks [202]. Effectively, the disparity in intensities and variances between peaks needs to be minimized in order for changes in low or high concentrated metabolite to make equal contributions to the PCA, PLS, or OPLS model. A range of scaling methods have been described [202] that emphasize different features of the spectrum and have different advantages and disadvantages.

The primary application of 1D ^{1}H NMR spectra is to characterize the global metabolomic changes. The large number of metabolites and the limited chemical shift dispersion in a 1D spectrum lead to severe peak overlap that makes metabolite identification very challenging. As a result, alternative methods have been developed or implemented to simplify metabolite identification. These approaches include 1D NMR methods such as selective total correlation spectroscopy (TOCSY) [204, 205], and statistical methods such as statistical total correlation spectroscopy (STOCSY) [206] and ratio analysis nuclear magnetic resonance spectroscopy (RANSY) [207]. In addition, two-dimensional (2D) NMR experiments routinely employed to characterize natural products (^{1}H-^{13}C heteronuclear single quantum correlation, HSQC; 2D ^{1}H-^{1}H TOCSY) are also used for metabolite identification [159]. The 2D NMR experiments significantly reduce peak overlap by dispersing the chemical shift information into two-dimensions. Importantly, these experiments also improve the accuracy of peak identification by providing chemical shift information for correlated nuclei, such as ^{13}C-^{1}H and ^{1}H-^{1}H pairs in the 2D ^{1}H-^{13}C HSQC and 2D ^{1}H-^{1}H TOCSY experiments, respectively. Unfortunately, these NMR experiments are significantly more time-consuming than the simpler 1D experiment, requiring upwards of hours to complete. Also, ^{13}C-based NMR experiments require even longer acquisition times or the incorporation of ^{13}C labeled metabolites because of the low natural abundance (1.1%) and sensitivity of ^{13}C. However, the 2D ^{1}H-^{13}C HSQC experiment has a unique advantage when cells can be cultured with a ^{13}C labeled carbon source. Only a subset of the metabolome is highlighted and the ^{13}C label can be traced as it flows through specific metabolic pathways. Of course, this requires a judicious selection of the ^{13}C-probe to monitor the appropriate metabolic pathways.

2.6. Metabolomics Applied to PD

2.6.1. PD Biomarker Identification Using the Metabolome

2.6.1.1. Targeted Metabolomics Studies

An exciting potential of metabolomics is the identification of biomarkers to diagnose PD, to monitor disease progression, and to evaluate a patient's response to treatment. Thus, a primary goal of metabolomics is to identify or "discover" the specific metabolites significantly perturbed in response to a disease state. What are the metabolites that are biologically relevant or correlated with PD? Con-

versely, a traditional targeted approach follows changes to a few select metabolites based on prior knowledge or hypothesis. Correspondingly, targeted analysis has identified metabolite variations in CSF, blood and urine samples obtained from PD patients and animal models [208-212]. For example, Ahmed *et al.* [208] evaluated 22 targeted metabolites in plasma samples obtained from 37 healthy controls and 43 drug-naïve PD patients by using 1D ^{1}H NMR and multivariate analysis. The 22 metabolites were selected based on prior connections with PD as cited in the literature. Metabolites were identified using a combination of 2D COSY and TOCSY NMR experiments, and an NMR reference library for 292 metabolites to assign the chemical shifts observed in the 1D ^{1}H NMR spectra.

These metabolites were shown to incur statistically significant changes ($P < 0.05$; ANOVA) in PD plasma samples. Of the 22 targeted metabolites, 17 metabolites were decreased and 5 were elevated in PD patients (Fig. **5a**). The heat map depicts metabolite concentration differences between healthy controls and PD patients. The relative metabolite concentrations are indicated by a color gradient. Red indicates an increase in the average metabolite concentration and green a decrease. The metabolites with a decrease in concentration are suberate, methylmalonate, galactitol, citrate, malate, succinate, glycerol, isocitrate, ethanolamine, ascorbate, threonate, gluconate, acetate, trimethylamine, glutarate, methylamine and glucolate, while the five elevated metabolites are pyruvate, sorbitol, myoinositol, ethymalonate and propylene glycol. It is important to note that a typical heat map would normally contain all of the individual replicates instead of the group-wise average presented by Ahmed *et al.* By including all replicates, it would be possible to assess the within group variability and, critically, determine if all the patients from the diseased and normal groups cluster together. This would provide an important quality check to further substantiate the relevance of each of the 22 targeted metabolites to PD. Simply, is the metabolite uniformly increased or decreased across the 43 PD patients and 37 healthy controls? The data was also used to train an artificial neural network (ANN) for PD diagnosis. The resulting ANN had a classification accuracy of 97.14% and a 100% specificity. Importantly, this classification accuracy was computed on held-out samples.

A PLS 2D scores plot generated from the NMR data identified a clear separation between PD patients and healthy controls (Fig. **5b**). Unfortunately, no statistical validation of the class separation in the PLS scores plot was provided. Again, PLS is a supervised method, is inherently biased and will always show a separation in the scores plot regardless of the existence of any true separation between the classes [179]. The separation in the PLS scores plot is determined by the manually defined class designation, where the follow-up validation step determines if this observed separation is statistically significant. Thus, the PLS result is very difficult to interpret. Instead, PCA would have been a more appropriate choice to provide a direct assessment of the significance of the class separation between PD patients and healthy controls. PCA is unsupervised, does not use class designations, and, as a result, is inherently unbiased. Nevertheless, PLS is useful for identifying the spectral features and, correspondingly, the metabolites that primarily contribute to the class separation in the scores plot. Of course, any subsequent analysis is dependent on the reliability of the original PLS model.

Myoinositol, glucitol, citrate, acetate, and pyruvate were identified as the key contributors to the class separation in the PLS scores plot and, thus, may serve as potential biomarkers if the PLS model is valid. The observed increase in myoinositol may be indicative of a decrease in sciatic motor-nerve conduction velocity. It is well-established that diabetic neuropathy in both humans and

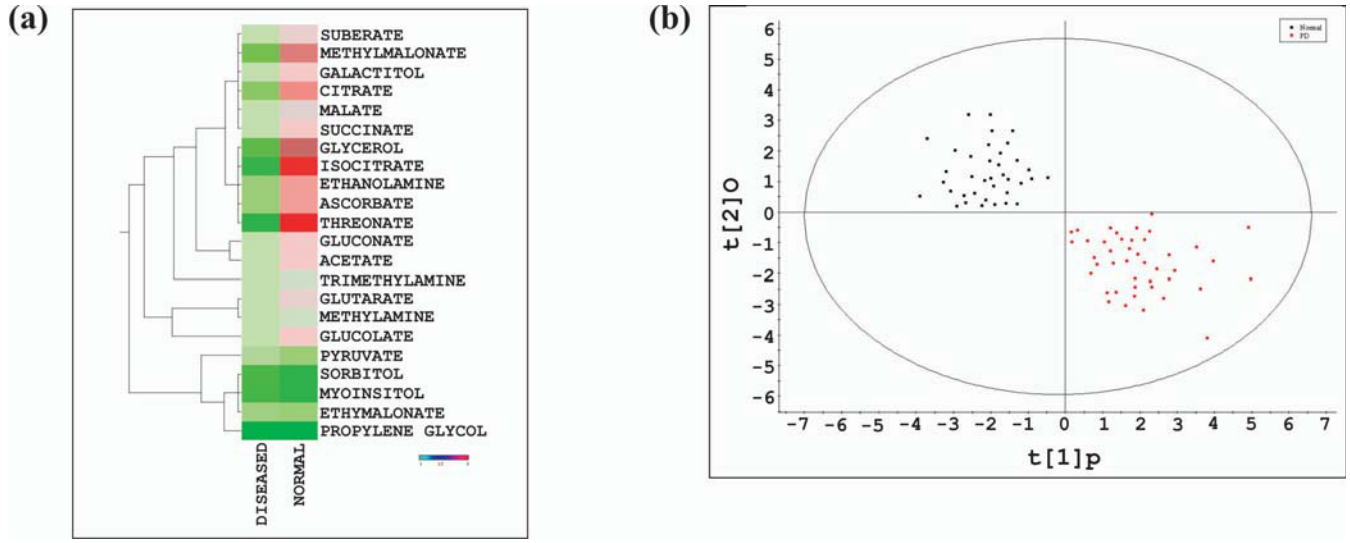

Fig. (5). (**a**) Heat map differentiation of metabolite. Average metabolite variability of blood plasma between PD patients (n = 43) and healthy controls (n = 37) are shown. Cluster analyses of the 22 differentially altered metabolites are selected based on significance P value ($P < 0.05$). The heat map depicts high (red) and low (green) relative levels of metabolite variation. (**b**) Partial least square discriminant analysis. PLS scores plot showing a significant separation between control subjects (n = 37) and unmedicated PD patients (n = 43) using complete digital maps. The observations coded according to class membership: black square = controls; red square = PD patients. (Reprinted with permission from reference [208], Copyright 2009 by BioMed Central Ltd.).

animal models is observed through the slowing of nerve conduction [213], which is also correlated with an increase in plasma and urine myoinositol levels [214, 215], and a corresponding decrease of myoinositol in nerve cells [216]. As a result, a complex metabolic mechanism has been proposed that correlates this myoinositol defect with the slowing of nerve conduction in diabetes [216]. This pathogenic scheme includes the polyols pathway, Na^+-K^+-ATPase activity, protein kinase C activity and phosphoinositide metabolism. The increase in sorbitol in PD may result from oxidative stress. The change in both myoinositol and sorbitol also implies a dysfunction of the polyols metabolic pathway and, correspondingly, a malfunctioning mitochondrion. Pyruvate (increased) is an end product of glycolysis that then enters the tricarboxylic acid cycle (TCA cycle) as acetyl-coA, where the other TCA metabolites citrate, acetate, succinate and malate were decreased. These results suggest an abnormal activity in pyruvate dehydrogenase. Interestingly, a differential down-regulation in the PDHB gene that has pyruvate dehydrogenase activity is also consistent with the observed increase in pyruvate levels and a decrease in TCA metabolites in the plasma of PD patients [208]. Correspondingly, Ahmed *et al.* suggests that pyruvate plasma levels may be a diagnostic for PD. Of course, given pyruvate's central role in metabolism, it is highly unlikely that pyruvate will be a unique biomarker for PD since pyruvate will probably be affected by other diseases besides PD.

Unfortunately, multiple discrepancies have been reported in the literature for metabolites associated with PD (Table **1**). Importantly, the magnitude of these discrepancies can be misleading since different sets of metabolites were targeted by different studies. For instance, none of the 22 metabolites described above (Fig. **5**) and the focus of the study by Ahmed *et al.* [194] were selected by the four other studies summarized in Table **1**. Nevertheless, there are still differences in the identity of the metabolites; and the magnitude and direction of the concentration changes do differ between some of these existing studies. These contradictory results may be attributed to the different analytical techniques (LCECA, GC-MS) employed by these studies. This also leads to variations in sample preparation procedures, storage conditions, and sample sizes. Moreover, differences in the selection of study participants (random, age/sex matched, family member inclusion), variations in the disease phenotype (various PD genetic variants), disease progression, and disease treatment all potentially play a major role in the inconsistency of the identified potential biomarkers. Furthermore, as discussed previously, there are multiple factors contributing to the etiology of PD that includes age, exposure to pesticides and herbicides, and head trauma. A different set of metabolite biomarkers are potentially associated with each etiology. Finally, a variety of other factors such as diet, life-style, and comorbidity could create a complex background and mask metabolites associated with PD. Correspondingly, the design of the study and how these various factors are controlled or normalized may explain or contribute to the observed discrepancies (Table **2**).

Table 1. Summary of Metabolite Changes from Targeted-metabolite PD studies.[a]

	CSF			Plasma		
Metabolites	**Tohgi *et al.* [209]**	**Jiménez-Jiménez *et al.* [210]**	**Mally *et al.* [211]**	**Jiménez-Jiménez *et al.* [210]**	**Mally *et al.* [211]**	**Weisskopf *et al.* [212]**
alanine	-	n.t.	↓	n.t.	-	n.t.
arginine	-	n.t.	-	n.t.	↓	n.t.
asparagine	-	-	-	↑	-	n.t.
aspartate	↓↓	n.t.	n.t.	↓	n.t.	n.t.
GABA	↓↓	↑	n.t.	-	n.t.	n.t.
glutamate	↓↓	-	↓	-	-	n.t.
glutamine	-	-	↑↑	↑	-	n.t.
glycine	↓	-	-	↑	-	n.t.
isoleucine	n.t.	n.t.	↓	n.t.	-	n.t.
lysine	n.t.	n.t.	↓	n.t.	-	n.t.
methionine	n.t.	n.t.	-	n.t.	↓	n.t.
uric acid	n.t.	n.t.	n.t.	n.t.	n.t.	↓
valine	n.t.	n.t.	-	n.t.	↑	n.t.

[a] ↑ - increase ($P<0.05$), ↑↑ - increase ($P<0.01$), ↓-decrease ($P<0.05$), ↓↓- decrease ($P<0.01$), - no change, n.t. – not targeted.

Table 2. Summary of Participants in PD Metabolomic Studies

	Targeted Studies					Untargeted Studies		
	Tohgi *et al.* [202]	**Jiménez-Jiménez *et al.* [210]**	**Mally *et al.* [204]**	**Weisskopf *et al.* [212]**	**Ahmed *et al.* [208]**	**Bogdanov *et al.* [206]**	**Johansen *et al.* [217]**	**Michell *et al.* [218]**
PD Patients								
No. PD patients[a]	28	31	10	84	43 (15,13,15)	66	53 (41,12)	23
Gender (Female/Male)[b]	n/a	20/11	3/7	n/a	6/9 5/6 7/8	27/39	16/25 7/5	23/0
Age (years)[c]	59.8 ± 8.9 60.1± 8.8	62.6±12.5	65.9 ±63	71.5	58.2±11.7 60.2±11.2 55.7±12.1	66.0±11.1	64.8 61.1	68.6±7.2
PD treatment[d]	L-DOPA (18)	various (26)	0	n/a	n/a	various (51)	n/a	various (15)
PD Duration (years)[e]	n/a	7.0±6.0	6.6±1.1	n/a	n/a	7.1±4.9	11.2;11.7	2.77
Age at onset	n/a	56.6±12.0	n/a	n/a	n/a	n/a	n/a	n/a
Healthy Controls								
No. Healthy Controls[f]	22	45	10	165	37	25	15	23
Gender (Female/Male)[g]	n/a	27/18	7/3 3/7	n/a	16/21	17/8	7/8	n/a
Age (years)[h]	n/a	57.8 ± 15.4	57±10 55±7	n/a	58.5±11.8	61.5±12.2	66.4	n/a

[a]For the Ahmed *et al.* study, the PD patients were divided into three different groups corresponding to PD-Stage 1, PD-Stage-2 and PD-Stage 3. The number of participants in each group is listed in the parenthesis in that order. For the Johansen *et al.* study, the PD patients were divided into two groups corresponding to idiopathic PD and LRRK2 (PARK8) PD. The number of participants in each group is listed in the parenthesis in that order.
[b]The number of Female/Male participants in each of the three groups from the Ahmed *et al.* study and the two groups from the Johansen *et al.* study are listed in the same order as in the number of PD patients.
[c]For the Tohgi *et al.* study, the age distribution are listed for patients on PD medication and not on PD medications, respectfully. The age distribution for each of three groups from the Ahmed *et al.* study and the two groups from the Johansen *et al.* study are listed in the same order as the number of PD patients.
[d]The number of participants receiving a PD treatment is listed in parenthesis.
[e]The PD duration for the two groups from the Johansen *et al.* study are listed in the same order as the number of PD patients.
[f]The healthy controls from the Weisskopf *et al.* and Michell *et al.* studies are matched cases.
[g]The number of Female/Male participants is divided into two classes for the Mally *et al.* study based on contributions of plasma or CSF samples, respectfully.
[h]The age distribution of the participants from the two classes for the Mally *et al.* study based on contributions of plasma or CSF samples, respectfully.

As an illustration, Tohgi *et al.* [209] reported a dramatic reduction in the concentration of aspartate, glutamate and γ-aminobutyric acid (GABA), and a modest, but significant reduction of glycine in the CSF from PD patients compared to healthy controls. However, Jiménez-Jiménez *et al.* [210] demonstrated that PD patients had similar CSF glutamate, glutamine, asparagine and glycine levels. CSF GABA levels in PD patients were still higher than in healthy controls. Alternatively, plasma glutamine, asparagine, and glycine levels were higher, aspartate levels were lower, and glutamate and GABA levels were similar in PD patients relative to healthy controls. The results of Jiménez-Jiménez *et al.* also showed an increase in CSF glycine levels and an increase in plasma aspartate and GABA levels after levodopa therapy; an increase in plasma glutamine levels after treatment with a dopamine agonist; and a decrease in CSF and plasma glutamate levels, and plasma aspartate levels after deprenyl treatment. Alternatively, Mally *et al.* [211] observed a highly significant decrease in glutamate levels, a slight decrease in alanine, lysine and isoleucine levels, and a significant increase in glutamine level in the CSF from PD patients. Mally *et al.* did observe a decrease in glutamate similar to Tohgi *et al.* Fewer metabolite changes were observed in the serum of PD patients, which included a decrease in the levels of arginine and methionine, and an increase in the levels of valine. Finally, Weisskopf *et al.* [212] observed a reduction in uric acid (a natural antioxidant) levels in the plasma of PD patients.

Importantly, the study design varied significantly across these four projects (Table **2**). There was a large variation in the treatments received by PD patients. Tohgi *et al.* included

PD patients that were either receiving L-DOPA or no treatment at all. Conversely, Jiménez-Jiménez *et al.* included PD patients receiving a variety of treatments, Mally *et al.* only included PD patients that were not receiving a treatment and Weisskopf *et al.* did not report patient treatments. Similarly, there was a ten year range in the average age of the PD patients between the studies, a high of 71.5 for the Weisskopf *et al.* study and a low of 59.8 for the Tohgi *et al.* study. A similar variation was observed in the number of participants. Weisskopf *et al.* included 84 PD patients and 165 healthy controls, while the Mally *et al.* study only included 10 PD patients and 10 healthy controls. The Weisskopf *et al.* study used matched case controls, where the others used random controls. Critically, the PD patients were not further classified into PD stage or phenotype in any of these studies.

2.6.1.2. Untargeted Metabolomics Studies

Untargeted metabolomics has been recently applied to PD in order to identify disease biomarkers and for the investigation of drug metabolites resulting from PD treatment [217-221]. Bogdanov *et al.* [219] identified approximately 2000 metabolites in plasma samples obtained from 25 healthy controls and 66 PD patients by using high performance liquid chromatography coupled with electrochemical coulometric array detection (LCECA). A resulting PLS 2D scores plot showed a statistically significantly separation ($P < 0.01$ by permutation test) for the metabolic profiles between healthy and PD patients. The main metabolites contributing to the observed PLS separation was an increase in 8-hydroxydeoxyguanosine (8-OHdG) and a decrease in uric acid and glutathione in the plasma of PD patients. Johansen *et al.* [217] applied a similar metabolomics strategy, LCECA combined with multivariate data analysis, to assess changes in plasma samples obtained from PD patients with the G2019S LRRK2 (PARK8) mutation. The study also enrolled PD patients without any known mutation (idiopathic) and asymptomatic family members of those PD patients with or without the G2019S LRRK2 (PARK8) mutations. Again, 2D scores plots from PLS depicted an obvious visual separation (no statistical significance was presented) between the metabolic profiles for LRRK2 (PARK8) mutation patients, idiopathic patients and control subjects. The metabolomic analysis also indicated an aberration in the purine pathway in PD. Michell *et al.* [218] investigated the metabolic profiles of serum and urine samples from 23 female patients with Parkinson's disease (PD) and 23 age and sex-matched controls using GC-MS, PCA and PLS. Contrary to the Bogdanov *et al.* [219] and Johansen *et al.* [217] studies, PCA or PLS ($P = 0.67$; χ^2 test) did not yield a separation between healthy and PD patients using serum samples. Furthermore, PCA did not yield a separation between healthy and PD patients using urine samples, but interestingly, PLS did ($P < 0.01$; χ^2 test). Importantly, the separation in the 3D PLS scores plot could not be attributed to any particular metabolite. This suggests that only a subtle difference exists in the metabolome between healthy and PD patients. These contradictory results could be attributed to the different experimental protocols and variations in the study designs (Table **2**). Ahmed *et al.* included PD patients from three stages of PD, while Johansen *et al.* recruited idiopathic PD patients or patients with the *LRRK2* (*PARK8*) PD variant. Again, there were variations in the number of participants (23 to 66 PD patients, 15 to 37 controls), average age range of the participants (55.7 to 68.6), and PD treatments.

2.6.2. PD Pathogenesis Investigation Using Metabolomics

Metabolomics is also an invaluable systems biology tool and an important approach for studying the underlying mechanisms associated with PD. Abnormal choline metabolism and mitochondrial electron transport system (ETS) dysfunction have been closely associated with central nervous system diseases like PD, but the relationship between ETS and choline metabolism is not well-understood. Towards this end, Baykal *et al.* investigated the relationship between functionally impaired ETS and choline metabolism using NMR metabolomics [222]. Human SH-SY5Y neuroblastoma cells were treated with a set of ETS inhibitors, where each compound selectively inhibited one of the five (I through V) ETS complexes. The extracted cellular metabolome were analyzed by 1D ^{1}H NMR and PCA. Metabolites of choline and phosphorylcholine were further quantified by matrix-assisted laser desorption/ionization mass spectrometer (MALDI-TOF MS). Each ETS complex inhibitor resulted in a separate cluster in the PCA 2D scores plot consistent with a distinct perturbation of the cellular metabolome. Thus, the inhibition of each ETS complex leads to a specific mitochondrial dysfunction, and a distinct phenotype. Nevertheless, all the ETS complex inhibitors resulted in a significant increase in choline and associated metabolites. This is consistent with the observation that choline is elevated in CNS diseases [223, 224]. The increase in choline may occur because the ETS complex inhibitors may also target choline metabolic enzymes as evident by an observed increase in the induction of choline kinase by all of the inhibitors.

Gao *et al.* [221] used 1D ^{1}H-NMR spectroscopy and PLS to detect metabolic changes in the striatum of 6-OHDA-induced Parkinson's rat. The right striatum of Sprague–Dawley rats were injected with 1.5 μg/μL of 6-OHDA. After the rats were sacrificed, specimens of both right (6-OHDA dosed) and left (control) striatum were dissected, followed by extraction of the metabolome for analysis by NMR. As expected, the resulting PLS 2D scores plot indicated a clear separation between the control and 6-OHDA treated groups. Unfortunately, the PLS model was not statistically validated making it difficult to interpret the reliability of the results. The corresponding PLS loading plot indicated that glutamate and glutamine were among the major contributors to the class separation. A statistically significant ($p < 0.05$) increase in glutamate and ɤ-aminobutyric acid (GABA), and a decrease in glutamine was also observed for the 6-OHDA treated striatum based on a comparison between normalized NMR integrals. These results suggest a likely shift in the steady-state equilibrium of the Gln-Glu cycle between astrocytes and neurons. A change in the Gln-Glu cycle would disrupt the balance between excitatory and inhibitory brain processes that would potentially lead to long-term abnormalities in glutamatergic and GABAergic activities. 6-OHDA induced perturbations in other cerebral metabolites (alanine, lactate, N-acetyl aspartate and taurine) that also suggest the possible involvement of energy metabolism and the TCA cycle in the pathogenesis of PD.

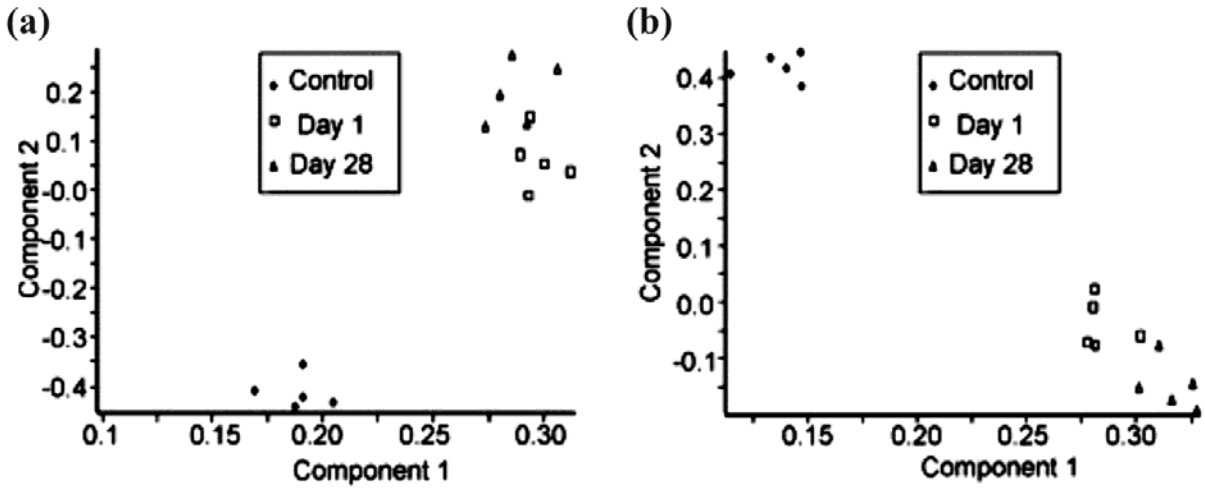

Fig. (6). The scores plot (**a**) from PCA analysis of the positive UPLC/MS data and (**b**) for negative UPLC/MS data for animals after day 1 and day 28 post-dosing of vehicle only (control) and 200mg/kg tolcapone administration. (Reprinted with permission from reference [220], Copyright 2009 by Elsevier).

2.6.2. PD Drug Discovery Using Metabolomics

In addition to biomarker discovery and mechanistic studies, Sun *et al.* [220] demonstrated the use of metabolomics to investigate drug metabolism in PD. During the evaluation of a potential new drug, it is critical to ascertain both the drug's efficacy and toxicity. Tolcapone, a catechol-O-methyl transferase inhibitor for PD treatment, was used to dose Sprague-Dawley rats. Urine samples were collected over a 28 day period to identify the metabolite profile. UPLC/MS, MS/MS (MS^2) combined with multivariate statistical analysis was used to investigate the impact of tolcapone on the urine metabolome. A total of 15 different metabolites of tolcapone were identified using both positive and negative modes of LC/MS and MS^2. While two of the observed metabolites, could be oxidatively bioactivated to induce liver toxicity, these reactive species were not observed and histopathology analysis indicated no significant changes. The PCA 2D score plots show complete separation between the control group, and the day 1 and day 28 dosed groups (Fig. **6**). Importantly, tolcapone and the metabolites of tolcapone were the primary contributors to this class separation. Also, there was a significantly larger separation between the control group and the day 28 dosed group. This is consistent with the accumulation of tolcapone and its metabolites from multiple doses.

3. CONCLUSIONS

3.1. Merits of Metabolomics for PD Research

Parkinson's disease is a CNS disorder with a high heterogeneity in clinical symptoms, with multiple etiological factors, and numerous, not well-understood pathological mechanisms. Metabolomics offers a unique opportunity to study this complex disease from a systems biological perspective. Changes in the metabolome are correlated with phenotypes, and are a direct result of alterations in protein and enzyme activities. Thus, metabolomics may provide critical information on cellular processes, molecular interactions and metabolic pathways associated with PD. Correspondingly, the electron transport system, choline metabolism, the Glu-Gln cycle, energy metabolism and TCA cycle have been implicated in PD from NMR metabolomics studies discussed in this review. Other highlighted metabolomics studies demonstrate the great potential of using NMR metabolomics to identify novel biomarkers for diagnosing PD, for personalized medicine, and for aiding in drug discovery and development. But, there are also clear challenges with the application of metabolomics to study PD.

3.2. Limitations of Metabolomics

The metabolomics studies presented in this review yielded contradictory results in the identification of metabolite biomarkers for PD. This unfortunate outcome is likely due to a combination of experimental errors, the use of different analytical techniques, the use of different targeted metabolites, the heterogeneity of PD, and different study designs. The appeal of metabolomics is the simplicity of the methodology, but it is also easy to obtain erroneous results. Simply, the metabolome is sensitive to any variation in experimental conditions.

The metabolome is a heterogeneous mixture of compounds with a range of turn-over rates, stabilities, solubilities, and volatilities. Thus, improper sample handling and preparation procedures may perturb the composition of a biological sample. For example, removing an extraction solvent like methanol may also result in removing other volatile metabolites. Also, residual enzymatic activity would affect the concentrations of associated substrates, co-factors and products. A biological sample is nutrient-rich, so a loss of sterility would also result in a dramatic change in the sample composition. Concentrating or drying a biological sample may result in the precipitation of specific metabolites due to limited solubility or changes in ionic strength or pH. Similar effects may result from choice of buffer or pH. The solubility of a metabolite can also be negatively affected by the presence of other metabolites in the sample. How long the sample is kept at room temperature, how long the sample is stored before analysis, and how many freeze-thaw cycles a sample undergoes are all factors that can perturb the composition of a biological sample.

Different analytical techniques (NMR, MS, IR, LCECA, etc.) have different strengths and limitations, and hence, emphasize different regions of the metabolome. Thus, it is not surprising that the application of various analytical techniques may also result in different outcomes. NMR is relatively insensitive technique and will only detect the most abundant metabolites, so a change in a low concentration metabolite would be unobservable by NMR. In contrast, MS depends on compound ionization and volatility. A number of metabolites do not produce a detectable molecular ion [225]. Moreover, the heterogeneity and complexity of the metabolome may lead to additional ion suppression and further diminish the detection of weakly ionizable metabolites. In addition, the application of chromatography to separate the metabolome may change the relative concentrations of a select set of metabolites in the MS spectrum. Again, the diversity of the physiochemical properties of metabolites will inevitably lead to a range of compound recoveries from chromatography columns; and correspondingly, result in relative changes in metabolite concentrations.

An observed difference (or lack of a difference) in the metabolome of biofluids from healthy and PD patients depends both on the disease and patient demographics. A patient's lifestyle, medical history, disease progression, medical treatments, gender, age, race, diet, etc. are all likely to affect their metabolome and may obscure the expected similarities between PD patients or between healthy controls. For example, a difference in the metabolome would be expected between PD patients receiving or not receiving a drug treatment. In fact, this difference may mask any similarities in the metabolome due to the disease itself. Similar issues arise if the PD patients have a variety of disease etiology or are at different stages of disease progression. The situation becomes even more complex when participants vary in sex, age, diet, lifestyle behavior, and comorbidity, to name just a few possibilities. Again, all these factors are likely to affect the metabolome and obscure any within group similarities. As a result, it becomes difficult to find a common difference in the metabolome between PD patients and healthy controls. In fact, any metabolite identified as a potential PD biomarker may simply be a result of a coincidence or from another prominent demographic besides PD. Thus, the inconsistency in the observed PD metabolite biomarkers (PCA, PLS, and OPLS) is not unexpected given the diversity in the patient demographics (Table **2**).

3.3. Potential Solutions and Future Directions

Validating and optimizing the experimental protocol is fundamental to a reliable metabolomics study [89, 134, 141-156, 162]. This is achieved by eliminating or minimizing all factors that cause undesirable changes in metabolite concentrations. In practice, this may be challenging, but general goals include minimizing the number of experimental steps and limiting sample handling, making the process as fast as possible, keeping the sample cold or frozen at all times, and analyzing the samples as soon as feasible.

The possibility that various analytical techniques may yield different outcomes for a metabolomics study can be turned into strength. As highlighted above, analytical techniques have different limitations and advantages, in essence, the techniques should be viewed as complementary to each other. Thus, a current trend in metabolomics is the integration of multiple techniques in a metabolomics study [123-125].

PD is a heterogeneous disease and the analysis of the metabolome to identify potential biomarkers requires a focused approach. Correctly addressing this challenge requires controlling or eliminating all patient variables besides the specific disease state. Ideally, all the PD patients should have the same etiology and stage of PD, and are not receiving any drug treatment. The participants should be the same age, sex, and equally healthy. Diet should be strictly controlled and alcohol consumption, smoking, and other medications should be eliminated. In effect, the only difference in the metabolome between the control and PD group should result from PD. Of course, this scenario could be very challenging to achieve in practice.

Also, observing a change in a metabolite between a control and PD group does not necessarily infer the discovery of a PD biomarker. The potential PD biomarkers need to be validated. One valuable approach is to simply repeat the study with a second group of participants and confirm the identification of the same set of metabolites. For the majority of the PD studies reported here, the number of participants in each class (healthy vs. PD) is too small (< 80) for statistical significance (α 0.05, power 90%), especially given the presence of other variables [226]. Also, a number of the models generated from supervised multivariate analysis were not validated [93, 180]. Similarly, the statistical significance of the defined group clusters in the PCA, PLS and OPLS scores plot are generally not evaluated [177, 227]. These are common occurrences in metabolomics studies, and are not unique to the PD field. Thus, the metabolomics community needs to adopt standard protocols for reporting the statistical validation of metabolomics data that also needs to be enforced by publishers [228]. The lack of validation in the PD studies reported herein does not necessarily negate the value of the individual outcomes, but it does raise a serious concern especially given the observed inconsistencies between the various PD metabolomic studies. Additionally, the significance of a change in a specific metabolite and its relationship to PD needs to be statistically verified [228, 229]. If a set of metabolites are proposed as a potential diagnostic test for PD, the statistical significance of the metabolite set also needs to be adjusted for multiple comparisons using the Benjamini-Hochberg, Bonferroni, Holm, or other similar methods [230, 231]. The observed metabolite biomarkers may not be unique to PD and may be associated with other CNS diseases, an immune response or some other comorbidity. Thus, the inclusion of multiple control groups, emphasizing a global analysis of the metabolome, increasing the number of participants, validating the multivariate models, and repeat studies using a second study group are all critical to a successful outcome from a PD metabolomics study. Metabolomics is still a relatively new field of study and, as a result, experimental protocols are still being developed and optimized. Correspondingly, the application and impact of metabolomics on PD has been limited to date. Nevertheless, as the methodology continues to evolve and improve, metabolomics has the potential of making significant contributions to PD research.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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