EXPERT
REVIEWSExploiting the potential of
molecular profiling in
Parkinson's disease: current
practice and future probabilities*Expert Rev. Mol. Diagn.* 10(8), xxx-xxx (2010)**George D Mellick^{†1,2,3},
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Parkinson's disease (PD) is a common, heterogeneous syndrome diagnosed clinically by the presence of classical neurological symptoms and the absence of 'red flags' that suggest alternative secondary parkinsonian disorders. Neuropathologically, nigrostriatal loss and the presence of proteinaceous inclusions (Lewy bodies) confirm the diagnosis. For PD, molecular profiling promises much but is yet to deliver in terms of breakthroughs for identifying at-risk individuals, detecting disease at early stages, improving diagnostic certainty, prognosticating future outcomes or providing surrogate markers of therapeutic efficacy. Recent, large-scale omics studies, driven by technological advances, have generated terabytes of data, but not yet met the goal of developing biomarkers suitable for clinical use in PD. In this article we critically evaluate the recent literature to identify the key roadblocks and realistic opportunities facing researchers interested in utilizing molecular profiling in the clinic to improve the diagnosis and treatment of PD.

KEYWORDS: biomarkers • genomic • metabolomic • molecular profiling • Parkinson's disease • proteomic • transcriptomic

Parkinson's disease (PD) is a common, complex and heterogeneous neurodegenerative syndrome affecting approximately 0.2% of people in the developed world [1,2], with an age-related incidence that increases from 0.3 per 1000 person-years in subjects aged 55–65 years, to 4.4 per 1000 person-years for those older than 85 years [3]. The worldwide prevalence is expected to exceed 9 million by 2030 [4]. Each individual patient with PD incurs an annual cost to their community (direct and indirect) in the millions of dollars [5,6]. A diagnosis of PD follows an insidious onset and progressive emergence of a constellation of classical clinical signs, including tremor, slowness of movement (bradykinesia), rigidity and postural instability. The classical diagnosis is reinforced by supporting features, such as an asymmetrical preponderance, a symptomatic response to dopaminergic agents and the absence of signs that might indicate an alternative akinetic rigid syndrome. For the purpose of this article we will use the term PD to refer to this classical symptomatic presentation for PD. Recently, there has been renewed interest

in the prodrome of PD, which occurs prior to the overt development of movement symptoms. This has an extremely variable presentation but may involve olfactory and autonomic dysfunction, rapid eye movement sleep disturbances, and neuropsychiatric manifestations, such as anxiety and depression [7]. Neuropathological examination (post-mortem) confirms a diagnosis of PD when degeneration of the nigrostriatal tracts in the basal ganglia is accompanied by the presence of the pathognomonic proteinaceous inclusions known as Lewy bodies. However, some familial parkinsonism syndromes, such as that associated with mutations in the parkin gene (*PARK2*), do not commonly demonstrate Lewy body pathology and others are yet to be fully characterized neuropathologically [8]. While the experience of expert movement disorder clinicians [9] and the benefit of well-characterized research diagnostic criteria [10,11] can increase the diagnostic specificity to more than 90% [12], the sensitivity and specificity of the clinical diagnosis by family physicians and general neurologists is relatively poor, particularly at the early stages when

neuroprotective interventions would be of greatest benefit. PD medicine is in desperate need of better ways to assist clinicians with diagnosis, prognosis, therapeutic monitoring and adverse-event predictions in their patients. Despite significant progress in recent years, such tools remain elusive. This article critically examines the current research landscape with regards to molecular profiling in PD. We will outline some recent advances, discuss the significant challenges facing the field and provide some realistic predictions as to the road ahead.

Definitions, scope of review & the framework of our literature search

The use of the term ‘molecular profiling’ in medicine emerged in the 1990s out of the cancer research literature, referring to the use of microarray technologies to assist with diagnosis, prognosis and treatment through the interrogation of gene expression at the mRNA level. Here we use this term more broadly to also encompass any content-rich source of molecular information (TABLE 1) derived from the omics technologies. An idealized representation of how the various layers of information in different omics datasets interact with each other and influence disease is presented in FIGURE 1.

These technologies, together with bioinformatics, are perceived to be at the vanguard in the battle against complex human disease, the development of clinically relevant biomarkers and the pursuit of personalized medicine [13]. We define biomarkers as characteristics that are objectively measured and evaluated as indicators of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (TABLE 1) [14]. There are several stages in the disease trajectory for which biomarkers would provide extremely valuable insight (TABLE 2). Screening markers would help to identify at-risk individuals, while diagnostic markers assist the clinician to more confidently arrive at a diagnosis (sometimes also referred to as ‘trait’ markers). There are also prognostic markers that help to predict future events and

progression (state markers), which monitor disease trajectory and the influence of interventions. In complex conditions such as PD, it is unlikely that one biomarker could fulfill all these objectives.

Literature search

We used the electronic resources of PubMed (US National Library of Medicine) to conduct several literature searches. A search of PubMed using the terms ‘biological markers’[Mesh] AND ‘Parkinson disease’[Mesh] yielded 575 results to 1 June 2010. Of these, 532 results related to articles in English published since 1990. A second search using the following strategy: ((“genomics”[Mesh] OR “proteomics”[Mesh]) OR “gene expression profiling”[Mesh]) OR “metabolome”[Mesh]) AND “Parkinson disease”[Mesh], yielded 125 results. Together these searches yielded 643 unique journal articles. Noticing that a large proportion (n = 101) of these articles were classified as ‘reviews’, we decided to concentrate our analysis on the articles published between 1 January 2008 and 1 June 2010 and the citations contained therein.

Current use of biomarkers in clinical diagnosis & management of PD

Olfactory dysfunction is a very sensitive non-molecular biomarker of PD [15–18]. PD patients almost always have significant olfactory loss compared with young healthy subjects and 75% of PD patients perform below age-adjusted norms [16]. While this suggests that olfactory function is a reliable marker of disease (with normal function sensitively ruling out a diagnosis of classical PD) the marker is non-specific. Formal, epidemiological specificity data are not available, but olfactory deficits are relatively common in several other neurological conditions, including Alzheimer’s disease (AD) [18].

At present, most laboratory chemical tests or morphological imaging strategies used in the Parkinson’s clinical workup are employed solely to exclude other neurodegenerative diseases [19,20].

Thus the impact of molecular diagnostics on the care of PD patients is currently limited [21]. A recent algorithm for clinicians on the classification of hereditary parkinsonism summarizes many of the suggested laboratory investigations and neurophysiology tests that may be employed in the diagnostic workup of the clinically uncertain Parkinsonian patient [22]. These include peripheral blood smears, serum biochemistry, erythrocyte antigen markers, cerebrospinal fluid (CSF) analysis, urinary tests, neuromuscular testing, nerve conduction studies, electroencephalography, polysomnography and ophthalmological assessments. The specifics of these techniques and their use in PD clinical practice will not be covered further in this article; suffice to say that these largely rule out the diagnosis of conditions that are not classical

Table 1. Definitions.

Phrase	Definition
Molecular profiling	The use of high-content data sources of molecular information (e.g., genomic, transcriptomic, proteomic, metabolomic) to develop a phenotypic descriptor of a biological state
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention
Genomic	Pertaining to genome-wide DNA sequencing and genotyping particularly used to describe single-nucleotide polymorphism genotyping data
Transcriptomic	Pertaining to mRNA expression information for a specific tissue under a particular set of conditions; usually determined using a microarray platform
Proteomic	Pertaining to the full complement of proteins extant in a tissue, bodily fluid or their extract
Metabolomic	Pertaining to the full complement of all low-molecular-weight molecules present in a tissue, bodily fluid or their extract

PD but that can manifest symptoms of parkinsonism as an accompanying feature. In addition, PD diagnosis can be assisted by the use of neuroimaging of the basal ganglia dopaminergic system using PET, SPECT and MRI techniques [23], transcranial sonography [24–26] and cardiac imaging using [^{123}I]-MIBG [27–30]. These techniques are becoming more routinely available; however, they remain largely restricted to the research environment. These imaging modalities may prove to be extremely valuable tools for the future development of molecular biomarkers for different aspects of PD. Currently PET and SPECT techniques are sensitive and specific enough to identify pre-motor PD [31–33], particularly in asymptomatic individuals who carry known, pathogenic *PARK* gene mutations, but they are far too expensive for universal routine clinical use. For good summaries of recent research into the development of these modalities in PD research, the reader is referred to a recent supplement to volume 24 of the journal *Movement Disorders* [34].

Figure 1. The inter-relationship between the various layers of omics information in health and disease. Perturbation at any of these levels may influence the trajectory from health to Parkinson's disease (PD). Information regarding the initial risk for disease, initiation of the disease process, progression and prognosis may be revealed in the genome, transcriptome, proteome and metabolome. Likewise normal ageing and the disease process itself results in changes at multiple levels. The complexity of PD pathogenesis means that any particular experimental regime will be limited in its scope but the integration of these multiple levels of information should enable the development of molecular biomarkers capable of effectively reflecting the various aspects of the disease outlined in Box 2.

Genetic testing

One current use of molecular profiling in PD clinical practice involves the increasing interest in the genetic testing of patients. The screening of patients suspected of having mutations in genes involved in familial parkinsonism syndromes [22,35] is becoming more common. However, in most clinical settings, such genetic analysis is not appropriate or indicated for PD patients and its utility is largely restricted to research settings or for patients with a specific familial clustering, early onset of disease or those in a family known to carry a pathogenic sequence variant in one of these genes [36,37]. In these circumstances, the testing should be accompanied by the level of genetic counseling appropriate for any Mendelian neurological condition [38]. This needs to be tailored to the individual patient's requirements, be made with full recognition that negative tests cannot exclude a genetic cause to the disease and the knowledge that, at present, there are no proven preventative or neuroprotective treatments available. These are particularly important issues given the increasing commercial availability of genetic tests screening these genes and the fact that, in some cases, these are now being directly marketed to the general public. Some guidance for clinicians in this area is provided by McNerney-Leo and colleagues [39] and the recent publication by a European Federation of Neurological Societies task force on molecular diagnosis in neurogenic disorders, including PD [40].

Requirements of effective biomarkers for PD

To develop effective molecular biomarkers for PD, we first need to consider what tissues may be the most appropriate and what we should be measuring in these tissues. As recently summarized by

Scherzer [41], Hennecke [42], and Schadt [43], in relation to gene-expression biomarkers, there are various models that can describe how a biological trait, measured in a tissue sample, may relate to an underlying disease process. These include the causal, reactive, independent and sentinel models [41–43]. In addition, Marek *et al* have recently outlined several important requirements of effective biomarkers for PD [44]. These include face validity, predictability and generalizability (Box 1).

Given the heterogeneous nature of PD, it is becoming clearer that one specific marker is unlikely to fulfill all of these requirements. It is more realistic that such markers will help us to better classify and understand the varieties of PDs, both etiologically and with respect to treatment response. Moreover, such markers will probably be different depending on what specific aspect of the disease process is being examined (Table 2). These issues present a paradox to the research community who are faced with the dilemma of selecting appropriate objective measures against which to test the suitability of any putative biomarker.

Tissue issues

To identify biomarkers in human neurodegenerative disease, three human tissue types have received the most attention: (post-mortem) brain tissue, CSF and peripheral blood. Each tissue source has its own relative merits with respect to the requirements for good biomarkers and these are summarized in Table 3. In humans, the use of brain tissue is usually restricted to post-mortem studies, which is not a clinically practical tissue for a routine biomarker assay. The anatomical regions of the post-mortem brain being examined significantly affect the interpretation of results.

Table 2. Landmarks in the course of Parkinson's disease where biomarkers would be valuable.

Marker	Definition
Screening marker	A marker to screen for individuals who might be at increased risk prior to the development of symptoms
Diagnostic marker	A marker to complement neurologic examination to aid diagnosis
Prognostic indicator	A marker that correlates with the probability of a specific clinical end point
Progression marker	A marker that correlates with the progression of the underlying pathology
Adverse-events indicator	A marker that assesses the risk for adverse events (such as treatment-related side effects)

Therefore, studies on brain tissue can be used in discovery science to establish baselines, regional variation or differences in case–control settings that can be further pursued in investigations using more easily accessible biofluids that act as surrogates and sentinels, reflecting processes ongoing in the brain. This has been well articulated as the sentinel hypothesis in recent reviews [41,45,46]. The sample quality of brain tissue depends on variables such as the duration and nature of agonal state, the post-mortem interval, sample preservation and preparation. As a result, human brain tissue is a relatively rare commodity and the number of studies using brain samples from repositories is accordingly small.

Cerebrospinal fluid, by its proximity to and intimacy with the tissues involved in the actual neurodegenerative process, is a very attractive biological fluid for biomarker investigation in neurodegenerative disease. It poses less practical and ethical considerations than post-mortem brain tissue, although it is more difficult to access compared with blood products. In addition, some specific caveats for the use of CSF for protein studies apply, as there is a marked difference in concentrations between blood and CSF (200-fold) [47]. Contamination of a CSF sample with blood is, therefore, a serious source of experimental error that is often underestimated. There are also recognized protein concentration gradients in CSF, that demand attention to ensure comparable information is collected between studies. These problems result in a relative paucity of studies of proteomic biomarkers derived from CSF. A careful consideration of these issues is exemplified in the recent publication by Hong and coworkers, who were studying the levels of the PD-related proteins, α -synuclein and DJ-1, in the CSF of patients with PD, AD and controls [48]. They considered the potential effects of both rostrocaudal gradient and blood contamination in their analysis. Hemoglobin levels in their CSF samples ranged between 0 and 9000 ng/ml. Given that both α -synuclein and DJ-1 have relatively high blood to CSF concentrations, separate analyses were performed following the exclusion of CSF samples with hemoglobin greater than 200 ng/ml. Interestingly, excluding the blood-contaminated samples revealed a statistically significant decrease in CSF α -synuclein levels in PD ($p = 3.4E-7$) that was not apparent when the contaminated samples were included in the analysis ($p = 0.56$). Correlations between CSF α -synuclein and DJ-1 levels were also substantially improved following exclusion

of the blood-contaminated samples. The finding of reduced CSF levels of these proteins in PD cases relative to controls or AD patients may prove to be a useful diagnostic biomarker if subsequently confirmed.

Blood (and its constituents) represent by far the most practical and accessible source of materials for biomarker development. It is a convenient choice of tissue that potentially reflects disease-related changes in mRNA transcripts, proteins and metabolites. Practical and ethical considerations are minimal and availability of the resource is not a concern. However, blood may not be in direct contact with the degeneration

processes in the brain. Anatomical structure, such as the blood–brain barrier, can act as obstacles to the transport of molecules into the blood from their origin in the degenerating brain. The relative distance of blood from the topological location of the major lesion may also make it difficult to detect these markers against the background abundance of other molecules. Other accessible fluid sources, such as urine or saliva, may also have some utility for identifying metabolomic biomarkers, although omics studies on these are yet to be published in the PD area. Human tissues to be used in the pursuit of biomarker development are not restricted to brain, CSF or blood cells. Assuming that disease-induced biological changes may be reflected in distant topographic locations of the body, cell lines such as fibroblasts, induced pluripotent stem cells (iPS) and their progeny [49] or cell lines derived from olfactory tissue [50] may also be potential candidates for analysis by omics technologies. These cell lines have not received extensive attention in this research space yet and are, therefore, not considered further in this article.

The developing field of molecular profiling in PD

The enormous technological advances of recent times have seen a movement toward a new era of discovery science. The suffix, 'omics', originally derived from the word 'genome' pertaining to the 'collective' set of genes in the human chromosomes, has now become widely used to describe holistic approaches to generate extremely extensive and rich sources of information covering the entirety of a particular type of variable (TABLE 1) [51]. In the following section we summarize the contribution of omics technologies to the developing field of molecular profiling in PD.

Genomics & PD

While PD was once regarded as the archetypal non-genetic disease, we are now aware of several specific Mendelian forms and a sizable familial component of risk among the sporadic forms [52]. Traditional genome-wide linkage analysis in rare families with inherited Parkinsonism (showing classical Mendelian patterns) has successfully revealed mutant proteins involved with clinical phenotypes largely indistinguishable from idiopathic PD. There have now been over 500 distinct DNA variants in five genes associated with familial Parkinsonism; these include

simple mutations and copy number variations (CNVs) [53]. This research space has been recently extensively reviewed by many others [22,37,54–56] and will not be further discussed here.

Genetic linkage

Similar genetic approaches to hunt PD genes in larger groups of small unrelated families have been less successful (for examples see [57–60]). This outcome is variously attributed to the genetic complexity of PD *per se*, with multifaceted reasons involving genetic and allelic heterogeneity, complex polygenic factors, epistasis and gene–environment interactions being involved.

Genome-wide association studies

Much expectation was placed on the outcomes of genome-wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) to identify genetic variables associated with increased risk for disease. The first forays into these experimental approaches [61,62] yielded a number of candidates that failed to replicate in subsequent large-scale follow-up studies [63,64]. These studies were not sufficiently powered to detect variables with the modest effect sizes thought to be relevant to idiopathic PD. Despite these limitations, similarly powered GWAS have subsequently pursued investigations into SNPs associated with age-of-onset for PD [65] and susceptibility to familial PD [66]. The value of these studies remains to be determined.

Recently, a second generation of more appropriately powered large-scale GWASs in PD have been published [67,68]. These provide important confirmation that SNPs around the familial Parkinsonism genes α -synuclein (*SNCA*), *LRKK2* and *MAPT* do associate with PD and identified two new potential risk loci (*BST1* and *PARK16*). While this proves the existence of risk-altering common genetic variables, their modest influences on the risk for PD further justifies the recommendation that pre-symptomatic genetic screening for PD risk in unaffected people is unwarranted at this time.

Transcriptomics & PD

The study of gene-expression profiling in PD is a rapidly developing area. Initially concentrating on microarray technologies, research is now moving into newer resequencing approaches that provide a more comprehensive coverage of the diversity of transcripts in various tissues. It is now worth asking the question, “How much have transcriptomics contributed to the ongoing search for useful PD biomarkers, and what can we expect from this technology?”

Generating expression profiles of mRNAs (between different tissues or under specific experimental conditions, such as in health and disease) can provide extremely valuable information about

Box 1. Biological traits associated with a specific disease process in the body must follow one of several models and fulfill important criteria in order to be a useful biomarker.

Biomarker models

Causal model	Markers that directly reflect a physiological process involved with disease causation
Reactive model	Markers resulting from the body's response to the already established disease process
Independent model	Markers that are not causal in nature but reflect a process that accompanies or is coincident with the disease
Sentinel model	Markers in a tissue peripheral to the disease process but that flag the disease process

Requirements for effective biomarkers

Accessibility	Can the marker be readily acquired in the clinic and applied to a wide variety of patient settings?
Face validity	Is the marker meaningful or relevant to the disease process?
Predictability	Does the diagnostic marker predict disease in individuals who are not yet diagnosed?
Generalizability	How does the biomarker vary with stage of disease, age, sex, medications, or environment?

Data from [41–43].

cellular responses to particular stimuli and their specific environmental contexts. However, several points need to be taken into consideration:

- The results of gene-expression studies are particularly dependent on the specific question being asked in the experiment. They rarely distinguish between the differences in mRNA levels that are causal or reactive in nature with regards to their relationship to PD;
- The source of biological material for the gene-expression determination is of crucial importance (see above, tissue issues). The challenge is to understand how robust these differences are, and to correctly interpret what aspects of the disease these profiles are marking. The measured mRNA levels of transcripts (assayed in traditional microarray experiments) do not reflect the sum total of all regulatory cellular processes in the assayed tissue even at the RNA level. Multiple transcripts exist for each gene and the complexity of the transcriptional landscape has not been fully considered in transcriptomics of PD to date;
- Post-transcriptional events on the protein level are not detected via transcriptomics, necessitating additional analysis at the proteomic level before probable candidates for biomarkers can be identified;
- There are still concerns regarding the reliability, reproducibility and comparability of gene-expression studies across different experimental platforms [69–71]. Some of these concerns have been addressed in the MicroArray QualityControl (MAQC) project [72–74]. The technical performance of microarrays as assessed in the MAQC project provides some standardization

of data reporting and the use of common analysis tools. This provides a resource that represents an important first step toward establishing a framework for the use of microarrays in clinical and regulatory settings.

Overall, datasets generated by transcriptomic methods are voluminous, multidimensional and complex; care and attention is warranted with regards to their interpretation. Traditionally, results have been communicated as gene lists of the most differentially expressed transcripts between experimental groups (e.g., PD vs control), with little obvious concordance observed between the lists published from similar experiments. For the purposes of publication, discussion is often restricted to the most significant differences and interesting findings aligned with the experimenters' interests or arguments. Interrogation of the data using more global statistical approaches (such as pathway analyses) may improve concordance between studies and identify more appropriate targets for replication. For example, we recently carried out a pathway-based re-analysis of all PD transcriptomic studies from human tissue [75]. While the initially communicated gene lists from the published articles revealed no apparent concordance to one another, re-analysis of the raw data using a common pathway-based approach revealed substantially more consistency in the different datasets. As part of this analysis we were able to compare different areas of the PD post-mortem brain with similar areas in unaffected controls. Our work highlighted that the transcriptional profiles of the PD brain could demonstrably reflect the considerable loss of specific neuronal populations in the PD substantia nigra (SN); this cell loss was driving the observed expression differences in dopamine signaling pathways between PD and control SN. By accounting for this neuronal loss, we identified alternative differentially expressed cellular pathways in the PD brain [75]. Our re-analysis also revealed very few similarities between the differentially expressed gene pathways identified in brain and blood [75]. However, case-control comparison of blood transcriptomics by Scherzer and colleagues did identify differential dopamine biosynthesis and signaling, and mitochondrial function in blood cells of patients with PD compared with healthy controls [41]. This study showed that the α -synuclein gene (*SNCA*) and the *ALAS2*, *FECH* and *BLVRB* genes form a block of correlated gene expression. These four genes are co-induced by the transcription factor, GATA-1, which occupies a conserved region within the *SNCA* intron-1 and induces a substantial increase in α -synuclein expression. Endogenous GATA-2 is highly expressed in SN and modulates

SNCA expression in dopaminergic cells [76]. Transcriptomic differences seen in various tissues needs more attention and further highlights the fact that transcriptomic profiles from different tissues can mark different aspects of the biology of the disease process: frank pathology; vulnerability; compensatory mechanisms; or a combination of all of these.

Our analysis [75] also examined a transcriptome dataset from Cantuti-Castelvetri *et al.*'s PD case-control study of dopaminergic neurons from the SN, obtained via laser capture microscopy (LCM) [77]. This revealed few disease-specific differences, in agreement with the original report [77]. A subsequent study reported considerable differences in gene expression between residual dopaminergic neurons from the SN in the PD brain compared with those from controls, particularly in the *PARK* genes [78]. Comparing the information from these two publically available datasets, using a common analytical method, we found virtually no overlap at the gene or pathway level [SUTHERLAND GT & MELICK GD, UNPUBLISHED DATA]. There is an urgent need for further LCM-based studies, to resolve these discrepant results and to reconcile the differences observed between cellular, tissue and global gene-expression changes.

In summary, disease-related biomarkers, relevant to PD pathogenesis, duration, severity or therapeutic intervention by studies using transcriptomic approaches alone remain unavailable. The success in this area will necessarily depend on tightly controlled experimental questions and a more orchestrated means of facilitating replication studies that also complement this information with data from other experimental platforms, such as proteomics and metabolomics, in an attempt to seek convergence.

Proteomics & PD

Proteomics are a powerful tool to profile cellular peptide or protein contents in biological systems. As mentioned earlier, the study of transcriptomic changes does not give an accurate picture of the cellular environment at the protein level, as mRNAs may not be translated and proteins are subject to various post-translational modifications. Proteomic analysis can be seen as a seamless continuation of transcriptomics, although it is currently not possible to interrogate a protein for every mRNA identified. Proteomic technologies allow the determination of protein structure, localization and activities, generating a snapshot of a physiological or pathological environment. Generally, the high-throughput screening methods involve sample preparation, protein separation and protein identification/quantification (often involving mass spectroscopy [MS]) [79,80].

Brain studies

Only one comprehensive proteomic study of PD has been undertaken using post-mortem brain tissue comparing protein expression in human SN in controls and PD patients. Of 44 identified proteins, nine were found to be expressed differentially and the findings supported oxidative stress as a contributor to PD pathogenesis; mitochondrial and ROS-scavenging proteins were overexpressed in the PD brain [81]. As most results related to presynaptic SN proteins in afferent terminals, the authors suggested that modification of afferent fibers to the SN

Table 3. Tissue considerations for Parkinson's disease biomarkers: the relative merits of different tissue sources for the development of clinical biomarkers.

Tissue	Brain	CSF	Blood, urine, saliva
Accessibility	+	++	+++
Face validity	+++	++	+
+: Indicates the relative subjective merit +++ > ++ > +. CSF: Cerebrospinal fluid			

following degeneration of dopaminergic neurons may be causing upregulation of these proteins to increase dopamine release by nigrostriatal neurons.

A proteomic study by Pan and colleagues defined a proteomic fingerprint of normal human frontal cortex, identifying 812 different proteins in this tissue, including those purported to have been previously implicated in neurodegenerative disease [82]. Interestingly, less than 20% of the proteins detected in the cortex can also be found in CSF, highlighting the challenges we are faced with when considering surrogate tissue sources. Moreover, there was little overlap in proteins identified in the SN and cortex data. Again this highlights the difficulties in comparing findings across studies and different anatomical regions.

Recently, proteomics has also been applied to an examination of the Lewy body itself [83]. Cortical Lewy bodies in patients' tissue were enriched using a sucrose gradient preparation and compared with control samples without Lewy body pathology. The authors revealed approximately 40 proteins that copurified with the inclusion bodies, among them α -synuclein and UCH-L1, but more interestingly, a number of ubiquitinating or phosphorylating kinases, such as MAPKK1/MEK1, protein kinase C or doublecortin-like kinase, that may be participating in the modification of α -synuclein and other Lewy body components.

Another potential application of proteomics in PD biomarker discovery research is the area of mitochondrial proteomics. However, as yet, there has been no publication of a comprehensive analysis of the human mitochondrial proteome in relation to PD. The possibilities of this interesting research area have been discussed recently in a review by Pienaar and coworkers [84].

CSF studies

Proteomic profiling of CSF has been used in an attempt to develop biomarkers for PD. Zhang *et al.* [85] recently tested the performance characteristics of a multi-analyte profile (MAP) developed from their previous discovery CSF proteomics experiments [73,86]. They assayed a panel of ten proteins using a multiplex immune bead-based approach. Using a random forest clustering algorithm, they assessed the extent to which each of the ten proteins, age and sex contributed to a correct classification of samples into the appropriate clinical groups. The best discrimination was achieved by combining eight of the proteomic variables (Box 2); this correctly discriminated between 95% of PD and control subjects. Correct classification of AD samples was more modest at 75%. The combination of such potential biomarkers must be considered a logical step in the absence of single, more potent discriminators, until more of these are identified. Interestingly, none of these proteins was among those identified in the CSF proteome analysis of Sinha *et al.* [87]. These workers use 2D gel electrophoresis and MALDI-TOF, liquid chromatography (LC)-MS to look for differentially expressed proteins between PD cases and controls; they identified six putative biomarkers (Box 2). Again, as in the case of the transcriptomic studies, there is a notable lack of concordance in the results between the Zhang and Sinha studies that requires consideration and explanation. There are obvious and considerable methodological differences between these studies, which go

some way into explaining the discrepancies, but one might expect some common themes to emerge if significant disease-associated differences do, indeed, exist.

Blood proteomic findings in PD

One recent systematic analysis of the complete blood serum proteome has brought together a large amount of research into blood-derived proteomic biomarkers for neurodegenerative disease, including PD [88]. These workers first used multivariate discriminate analysis to identify 57 candidate proteins that could discriminate between retrospectively banked serum samples ($n = 469$), drawn from patients with different diagnoses, including AD, PD and amyotrophic lateral sclerosis (ALS). These proteins were grouped into five classes: cell degeneration biomarkers; haptoglobin proteins; inflammatory proteins; albumin proteins; and unknown proteins [88]. These candidate markers were then tested in a prospectively collected group of 62 PD patients and 30 controls recruited from two sites. In this validation step, 21 of the candidate proteins contributed significantly to the optimal discrimination between PD and control samples, with a resulting sensitivity of 92.9% and specificity of 93.3% [88]. These 21 putative biomarkers are listed in Box 3. The results of further validation studies will determine how useful such combinatorial biomarkers might be in a clinical setting.

To some extent, the results of these global proteomic experiments loosely reflect alterations in oxidative stress, inflammation or degeneration pathways, which are also observed in more directed studies of protein changes in PD [81,89–92]. For example, Chen and colleagues [93] recently suggested that higher levels of IL-6 predicted future risk of PD. Another group found no abnormalities in this particular marker, but noticed associations between plasma mannan-binding lectin and TNF- α , and impairments in motor and cognitive function in PD patients [90]. Ferroxidases, such as ceruloplasmin, have also been implicated in PD etiology and have been associated with age-of-onset in one recent study

Box 2. Analysis of the cerebrospinal fluid proteome in different studies has failed to reveal consistent differences between Parkinson's disease and control samples.

Zhang <i>et al.</i> (2008): best discriminators	Sinha <i>et al.</i> (2009): differentially displayed proteins
<ul style="list-style-type: none"> • Tau • BDNF • IL-8 • Amyloid β-42 • β-2-microglobulin • VDBP • Apo AII • Apo E 	<ul style="list-style-type: none"> • Serum albumin precursor • Serum albumin chain-A • Hemoglobin-β fragment • Mutant globin • PRR14 • Serum transferrin N-terminal lobe

While a combination of six CSF protein analytes was able to retrospectively discriminate between cases and controls with reasonable success in the study of Zhang and colleagues [85], none of these proteins was identified in the study of Sinha *et al.* [87].
BDNF: Brain-derived neurotrophic factor; VDBP: Vitamin D-binding protein.

Box 3. List of 21 putative proteomic biomarkers for Parkinson's disease.

- Chain A albumin mutant R218H protein
- Haptoglobin HP-2a protein
- X1 protein
- Complement factor I protein
- Apolipoprotein E3 protein
- Transthyretin 'dimer' protein
- Nucleoporin NUP 188 protein
- Haptoglobin HP-1 protein
- Albumin protein PRO2044
- Acidic histone H2A protein (PD/LBD)
- Apolipoprotein A-IV protein
- Transthyretin HYPE protein
- Complement C4b γ chain protein
- Chain A albumin mutant R218H protein
- Fidgitin protein I
- Immunoglobulin κ light chain protein
- Complement factor H/Hs protein
- Fidgitin protein II
- Albumin protein PRO2675 protein
- X2 protein
- Haptoglobin-related protein

This is a subgroup of 57 proteins uncovered by stepwise discriminate analysis as significant contributors to the discrimination between PD and control samples in a prospective investigation of 62 PD and 30 control subjects. Identified by Goldknopf and colleagues [88].

LBD: Lewy body dementia; PD: Parkinson's disease.

[94]. It is notable that global proteomic studies, to date, have not consistently identified other proteins strongly implicated in the pathogenesis of PD (such as α -synuclein) that have been extensively studied using more direct experimentation. Perhaps further technical development of the extraction and detection methods will improve the capacity of the next crop of proteomics studies to generate high-throughput data consistent with the more focused findings. In particular, given the huge dynamic range of protein concentration in blood, fractionating the serum or plasma to separate the high- and low-abundance proteins prior to comparative examination (a strategy being actively pursued in the cancer biomarker area) may lead to improved outcomes [95].

Metabolomics & PD

Metabolomics focuses on the identification and quantification of small molecules, or metabolites, in cells, tissues, and body fluids, and has been defined as the analysis of the quantitative complement of all low-molecular-weight molecules present in cells in a particular physiological or developmental state [96,97]. In essence complementary to transcriptomics and proteomics, the metabolome may also be seen as a kind of end point, which on a functional level, reflects transcriptional and proteomic changes in a cell in an amplified manner [98]. Metabolites are chemical solutes that can be analyzed using a range of chemical detection procedures (reviewed in [98]). Generally, the platforms used for large-scale metabolic fingerprinting include MS coupled to gas

chromatography or LC. However, nuclear magnetic resonance (NMR)-based metabolomics platforms are attractive as biofluids can be analyzed with little or no sample preparation [98,99]. LC-electrochemistry array (LCECA) metabolomics platforms are useful for analyzing neurotransmitter pathways and pathways involved in oxidative stress for both targeted and untargeted studies [100]. There have been some recent metabolomic studies in the Parkinson's area that have yielded interesting findings.

Bogdanov *et al.* characterized blood plasma metabolomic signatures in PD, using high-performance LC coupled with LCECA [101,102], in medicated and unmedicated PD patients and controls [89]. The aim was to identify diagnostic plasma biomarkers in PD. Their results showed that the antioxidant, glutathione, was significantly increased in PD patients, while urate (UA) levels were significantly decreased. The authors also identified oxidative DNA damage using 8-hydroxy-2-deoxyguanosine (8-OHdG) levels as an informative marker. Initially, unmedicated PD subjects were compared with controls to assess potentially confounding effects of symptomatic medications. In their analysis, the two groups could be completely separated and distinguished from each other. Using parameters from this first analysis, the authors could separate both medication-free patients and those taking dopaminergic therapies from normal controls [89]. In a recent interesting follow-up study, similar metabolomic profiling has been applied to individuals from families with PD caused by the genetic mutation in the *LRRK2* gene resulting in the G2019S substitution [103]. The study included 12 PD patients known to carry the disease-associated allele and 31 of their unaffected family members (21 of whom also carried the disease-causing allele). An additional 41 PD patients and unrelated healthy controls with no known genetic mutations were also profiled. A total of 712 analytes met the criteria for inclusion in the latent-structures discriminant analysis. The analyses could distinguish between: *LRRK2*-PD patients and controls; *LRRK2*-PD and idiopathic PD; *LRRK2*-mutation carriers with symptoms and those with no clinical symptoms; and asymptomatic *LRRK2*-mutation carriers and controls. The majority of the analytes driving the separations between groups remain unknown, but again, UA levels were lower in PD patients irrespective of mutation status and xanthine levels were lower in *LRRK2*-PD compared with controls. A number of these yet to be elucidated analytes may prove to be useful molecular biomarkers for PD.

Ahmed *et al.* tested whether PD-induced perturbations of the metabolome could be sufficiently characterized in plasma samples of healthy controls and drug-naïve patients with PD using proton NMR [104]. Multivariate data analysis was performed and followed by neural network pattern detection. There were 22 metabolites differentially expressed in PD (Box 4), 17 were decreased and five were elevated in patients. Pyruvate was the key metabolite contributing to the separation of PD patients from controls, identified by partial least-squares discriminant analysis (PLS-DA). This result was supported by gene-expression studies. Interestingly, neither UA or glutathione associated with PD in this study. Again, at face value, it is difficult to determine

the extent of consistent overlap in the results between these similar metabolomic studies. The face validity of the findings of Bogdanov and colleagues is enhanced by the fact that two of their identified discriminators, UA and glutathione, have well-established associations with PD. A large and growing body of evidence from clinical, epidemiological and laboratory-based studies suggests that there is a strong negative association between UA levels in blood and risk for PD [89,91,105–107]. Thus, to some degree at least, the identification of this analyte in Bogdanov's study serves as a positive control; it is disappointing, however, that this paper does not reveal the identity of the vast majority of discriminating metabolites that remain to be structurally elucidated.

Advantages & limitations of an omics approach to biomarker discovery

The platform technologies that make data-rich omics research possible are relatively recent additions to the scientist's armamentarium in the fight to increase our understanding of neurodegenerative disease and develop molecular biomarkers for complex conditions such as PD. Therefore, having outlined some of the recently published, data-intensive omics research studies in the PD field, it is worth reflecting on how these have impacted on the PD research landscape generally, what their advantages and limitations may be and where we are now heading.

The most obvious advantage of these technologies is their capacity for high throughput, thus enabling analyses at the scale of entire biological systems, such as whole cells, organs or other defined tissues. Simple identification of molecules, however, is insufficient to form a picture of the complex mechanisms and interactions in a biological system. The generation of huge datasets alone is not very meaningful or helpful if it does not identify already-known disease-related variables. So far, the data generated by omics studies have not closely paralleled the data collected in directed studies of gene transcription and protein changes observed in PD. This is astonishing in the face of the gigantic datasets that have been acquired. Moreover, few omics-derived molecular biomarkers to date have been successfully translated into clinical practice. To improve this situation, factors such as a lack of unified disease classification (and sample normalization), a poor consensus on thresholds of significance and comparison groups, the use of different techniques to monitor the level of transcripts or proteins, and the presence and use of different platforms need to be addressed. More nuanced analytical methods are required to better uncover the biologically relevant information from these large datasets. Reporting lists of the most differentially represented variables is unlikely to result in major advances. As mentioned previously, the employment of pathway approaches and combinatorial analyses may provide better options as the area moves forward.

Rules of evidence

In discovery-based research, such as the various omics approaches, very information-rich datasets are generated. In most cases, the experimental paradigm involves looking for discriminatory patterns in the data between two groups (in the situations mentioned

here, PD patients vs control subjects). It has been well acknowledged that such an approach has important limitations. In an excellent review of the topic, David Ransohoff has outlined some rules of evidence required for molecular marker discovery and validation in cancer research; these are equally relevant to all complex disease research, including molecular profiling in PD [108]. In particular, the issues of overfitting, irreproducibility and bias need to be considered carefully.

Overfitting can occur when the number of variables being used to discriminate between groups is large in comparison with the numbers of individuals in the groups being compared. This is often the case in the omics studies. For example, the largest PD omics studies published to date have many orders of magnitude more predictors than individual samples being compared. This leads to a high probability of identifying groups of variables that perfectly discriminate between groups purely by chance but with absolutely no relevance to the true outcome of interest. In fact, some epidemiologists suggest that to have confidence in the result, there should be on the order of ten-times more events (in this case, individuals in the groups being compared) than predictors (variables used to discriminate between the groups) [109]. In an attempt to mitigate for this important problem, tiered approaches and replication arms to experiments (where a validation set is used to test the discriminatory pattern identified in an initial training set) can be included in the studies. However, this has not necessarily been the case in the PD studies.

Box 4. List of 22 plasma metabolomic biomarkers.[†]

- Suberate
- Methylmalonate
- Galactitol
- Citrate
- Malate
- Succinate
- Glycerol
- Isocitrate
- Ethanolamine
- Ascorbate
- Threonate
- Gluconate
- Acetate
- Trimethylamine
- Glutarate
- Methylamine
- Glucolate
- Pyruvate
- Sorbitol
- Myoinositol
- Ethylmalonate
- Propyleneglycol

[†]Identified by Ahmed and colleagues as being associated with Parkinson's disease [104].

Five-year view

We are only just entering the era of functional genomics and systems biology in neuroscience [110]. This has already yielded novel insights into brain development, function and evolution. It is mind-boggling to imagine the impact of new technologies on the clinician's ability to diagnose, treat and monitor disease as we move forward.

For example, the advancement of DNA sequencing technologies means that affordable complete genome sequencing will soon be commonplace in both research and clinical settings. Traditional Sanger sequencing is being fast replaced as the gold standard by the sequencing-by-synthesis technologies [111] combined with methods for multiplex sequencing using paired genomic tags [112], both of which emerged around 2005. Advances in this next-generation sequencing has seen the two most utilized commercially available systems now producing hundreds of Giga bases of mappable sequence from more than 1 billion sequence reads per instrument run with further improvements to increase efficiency and reduce cost emerging all the time. Technologies enabling the sequencing of DNA from single molecules in real time, without the necessity for prior amplification (single molecule sequencing [SMS]), is also now available [113]. These advanced sequencing technologies will relegate the need for linkage-disequilibrium-based association analysis using SNPs and move transcriptomics from platform-based array methods, which assay specific transcripts, to full re-sequencing, enabling a more comprehensive surveying of the full diversity of gene transcription. Next-generation sequencing technologies will also impact upon genome-wide genetic CNV analysis, which has until recently focused on specific candidate genes, such as *parkin* [114] and *α-synuclein* [115,116], in PD research.

Moreover, these new technologies will profoundly influence our study of the epigenome, the non-sequence-related modifications to the genome that influence phenotype. Currently, genome-wide surveys of epigenetic changes, epigenomics, have not been published for PD. However, a number of preliminary studies have examined the methylation status of PD candidate genes, such as *α-synuclein* [117] and *MAPT* [118], in relation to neurodegenerative diseases. Interestingly, one advantage of the SMS sequencing technologies is that base modifications (e.g., methylations) can be directly assayed because the kinetics of polymerase activity is sensitive to these chemical differences [113]. This is set to revolutionize the study of epigenetics in the next few years.

The mapping of molecular events at an anatomical and temporal level in the living human brain in health and disease is not yet fully realized. Neuroimaging can achieve this to some extent, but this will probably remain too expensive to use at a population level in the near future. Nonetheless, large public resources now enable the researcher to examine regional gene-expression (transcriptomic) data across the entire adult [201] and developing human brains, and to compare and integrate this information with an expanding plethora of data generated in other species, most notably the mouse, for which genetic manipulation and targeted experimentation is possible. Over the next decade, integration of similar high-content data from diseased human brain will also emerge. Again, this highlights the importance of continuing to

foster and develop high-quality tissue banking efforts with accompanying high-quality clinical phenotyping. This will enable the type of multidimensional tissue analysis [119], from which robust molecular biomarkers will undoubtedly emerge. Initial efforts to integrate genomic SNP data and gene-expression levels across the human brain have already been published [120]. By integrating genome-wide expression (Illumina bead arrays) and genome-wide SNP data (Affymetrix chips) from the banked brain tissue of 200 neurologically normal individuals, Myers and coworkers recently reported that 58% of the known human transcriptome is expressed in human cortical tissue and that common genetic variability is correlated with this expression for over 20% of these transcripts [120]. Logical extensions of this type of study will correlate genomic data with anatomical region-specific or cell-specific transcript expression, as has already been published for the mouse brain [121]. Array technology is being replaced by next-generation sequencing approaches [110], which also allow for the study of RNA splicing, miRNAs, epigenetic modifications and novel transcripts, which are not readily assessable using the older technologies. There have also been early attempts to use brain-expression data to refine genome-wide and genetic-association information to find candidate genes for PD [122–124] and AD [125,126], an approach termed genomic convergence. Using this approach, Hauser and colleagues used serial analysis of gene expression (SAGE) to refine candidate genes for PD from regions identified in linkage experiments [122,124]. A list of over 3700 candidates was refined to 402 based on whether the gene was expressed in human SN [122], and further refined in a subsequent study [124] to 50 candidates based on those that were differentially expressed between PD and control SN.

In another whole-genome expression profiling study, the genomic conversion approach was used to identify gene candidates based on whether they were differentially expressed in SN neurons from PD patients and controls and associated with disease at a tagging SNP level [127]. Four possible biomarker candidates were identified: a NADH dehydrogenase (MTND2), a pyridoxal kinase (*PDXK*, vitamin B6/dopamine metabolism), *SRGAP3* (axon guidance) and *TRAPPC4* (vesicle transport). This study is a good example of how genome-wide expression studies in combination with association analysis can be used to identify potential genetic modifiers in neurodegenerative disorders from a larger number of candidates. The overlap of the genes determined in this and other studies is, however, limited to pathway disturbances that have been broadly implicated in PD, such as axon guidance or dopamine metabolism, and more functional studies are required to validate the results. However, the method has demonstrated potential value for determination of genetic risk factors. It is easy to see the power of further coupling this work with proteomic and metabolomic data derived from the same individuals.

Animal models in proteomics, transcriptomics & metabolomics

The next 5 years should also see a more concerted effort to integrate data from animal studies into the human studies searching for biomarkers relevant to PD. Animal models may serve as useful surrogates to investigate problems and aspects of neurodegeneration

that are not easily addressed in a human setting. Despite the limitations of animal models to exactly mirror human disease or interventional responses, they offer many advantages. These include accessibility to brain tissue for correlation and an easily manipulated and controlled experimental environment. A number of recent animal studies provide excellent complementary evidence for future human investigations. These studies can address more basic biological questions in specific settings with fewer confounding influences. For example, Scholz *et al.*, addressing the development of L-DOPA-induced dyskinesias in macaques, established a list of characteristic proteomic baseline marker expression levels distinguishing treatment-naïve, acute and chronic L-DOPA-treated animals [128]. These data pave the ground for future use of similar markers in humans as surrogates for the development of treatment-induced motor complications in PD. Another example is the use of genetically modified animals to provide insights into preclinical Parkinsonism and the effects of compensatory mechanisms central to early PD. These issues are not easily amenable to study in humans. A proteomic analysis of transgenic mice overexpressing human A30P α -synuclein showed a number of oxidatively modified proteins with an associated decrease of function [129]. Similarly, oxidation-induced mitochondrial damage has been a long-time potential culprit in PD etiology and animal models allow the study of proteomic changes following dopamine oxidation and the identification of uniquely susceptible mitochondrial proteins [130,131]. Old workhorses, such as the mouse MPTP model, can be used to generate surprising results using transcriptomics following novel treatment interventions, such as electroacupuncture [132], or administration of potential neuroprotective and disease-modifying drugs, such as rasagiline [133]. Important information is also being obtained from omics analysis of transgenic nematodes [134] and flies [135,136] that should assist interpretation of human data. The continued development of novel animal models [137] will also provide more opportunities to exploit, hone and coordinate the different layers of omics technologies to produce integrated data and a better picture of the concordant and discordant features of various neurodegenerative processes.

Conclusion

There is great and increasing potential for molecular profiling to enhance our understanding of PD and assist in the development of viable clinical biomarkers for neurodegenerative disease. To realize this potential we suggest that the following critical issues need to be foremost in researchers' minds:

Ask the appropriate research questions

Questions should be focused on well-defined parts of a biological system in well-defined homogenous populations and disease phenotypes. For example, biomarkers for disease risk and (early) diagnosis present at the very beginning stages of the disease process may prove difficult to detect in the current investigations of prevalent PD patient populations, which may be confounded by the effects of the disease that may have been active for some time. Questions regarding diagnosis, progression and prognosis need to be directly subjected to the omics approaches.

Improve the phenotyping of disease subgroups

There is very little doubt that the clinical diagnosis of PD may encompass a number of sub-phenotypes. These subgroups are currently poorly defined and generally not considered in omics molecular studies. Efforts should be made to generate more homogenous patient populations by grouping them according to various criteria, which would prove more informative for the identification of relevant biomarkers, such those that predict disease progression and prognosis, treatment responsiveness and predilection to certain symptom clusters.

Develop prospective studies of very early diagnosed cases & individuals at-risk for disease

The current situation is particularly unsatisfactory with regards to the absence of early diagnostic markers of PD that would potentially allow for therapeutic and disease-modulating interventions. Investigations in familial PD kindreds may offer the best opportunity to collect data relevant to the very early stages of PD (see [103] as an example).

Coordinate the data between technologies & approaches

Attempts should be made to make data acquisition and generation more comparable between research platforms and the various layers of high-content data. Orchestrated studies using the same biological samples are necessary to develop a firm understanding of how these different technologies influence results and their comparability.

Look for consistent thematic signals that emerge from the noise

At present there are terabytes of data that are noisy, difficult to interpret and apparently discordant. With further advancement of bioinformatics, researchers need to understand the sources of this noise, to minimize that due to technical error, to embrace that due to biological variation and to uncover the relevant parameters amenable to clinical exploitation.

With these issues in mind, it is possible to envisage novel molecular tools that will assist clinicians in the future diagnosis and management of people with PD.

Expert commentary

The investigation of molecular biomarkers for PD is a relatively new and rapidly moving research space. With the development of increasingly sophisticated technologies to comprehensively survey genes, gene transcripts, proteins and cellular metabolites – the so called omics technologies – it is worth assessing the progress thus far and the future potential for the current approaches. Initial omics investigations concentrated on direct comparisons between relatively small groups of clinically heterogeneous, prevalent PD patients and apparently healthy control subjects. Many of these early studies were driven by the technologies themselves rather than robust hypothesis-driven questioning. Large datasets were generated and group differences between cases and controls identified. However, *bona fide* biomarkers suitable for clinical application have not yet emerged. Nonetheless, there is cause for optimism with a number of promising leads still under follow-up

investigation. More focused experimental designs that integrate various levels of omics information at different stages of disease in well-characterized individuals, combined with the reducing cost of these technologies and an improved understanding of how to handle the huge information-rich datasets will, no doubt, lead to novel molecular biomarkers suitable for application in the neurology clinic of the 21st Century.

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Key issues

- Parkinson's disease (PD) is an important age-related neurodegenerative disease with a significant societal burden and an increasing worldwide prevalence.
- The impact of molecular profiling on the care of PD patients is currently limited.
- Biomarkers for: identification of at-risk individuals; diagnosis; prognosis; and treatment response are desperately needed
- The first forays into comprehensive omics-based strategies are yet to identify a molecular biomarker for clinical use in PD medicine.
- To improve the probability for success, the next phase of omics studies will need to:
 - Direct more focused questions to specific aspects of the disease process;
 - Improve the phenotypic evaluation of the subjects in the studies;
 - Develop prospective protocols to follow changes in individuals over time;
 - Coordinate technologies and approaches;
 - Look for consistent thematic signals emerging from the noise of the extensive datasets.

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