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Blood-based proteomics for personalized medicine: examples from neurodegenerative disease

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"Proteins in the blood serum can tell us what disease pathways and mechanisms of neuronal degeneration are active in patients."

Unbiased proteomic discovery

In 1927, the great Danish physicist Niels Bohr proposed that some natural phenomena can only be completely understood by combining two or more experimental approaches that cannot be simultaneously implemented. His student, Max Delbrück, applied this 'principal of complementarity' to biology, indicating that a full understanding of the role of biological molecules, such as proteins, requires the study of how they behave with other proteins, but at the level of the whole living organism.

Unbiased clinical proteomics operates from this uniquely comprehensive perspective, beginning with the patient. An unbiased monitoring of patient proteomes for disease-related changes provides results that have broad and deep implications that liberate a new paradigm to unlock the power of personalized medicine. Upfront we are seeking changes in proteins that can be monitored in a live patient (e.g., in blood serum) as biomarkers for diagnosis, differential diagnosis, patient monitoring, for selection of treatment options, and for new drug target discovery.

My first exposure to an unbiased approach to proteomics started early in 1972, when I was being recruited as a 'post-doc' into the 'Nuclear Protein Group' in the laboratory of the late Dr Harris Busch, MD, PhD, Professor and Chairman of Pharmacology at Baylor College of Medicine (BCM). Dr Busch took me up to an empty shell that was the seventh floor of the Jewish Institute for Medical Research at BCM and said "This is where we will put the Nuclear Protein Laboratory, where we will find proteins that are the keys to cancer."

In the ensuing years, under Dr Busch's relentless leadership and focus, the group, also including Mark OJ Olson, Larry R Orrick, Lynn C Yeoman, Charles Taylor, Raghu Ballal, S-I Matsui, Larry Rothblum, PK Chan, Mariana Yaneva, Egon Durban, Ierach Daskal and others, applied unbiased proteomics, using 2D gel electrophoresis, to what would now be referred to as the cell nucleolar and cell nuclear proteomes [1–3]. I was blessed to be a part of this effort and to recieve the benefit of their expertise for my project, which became the discovery, identification and sequencing of the first, previously unknown, cancer-related nuclear protein, using the proteomic technology of 2D gel electrophoresis: protein A24, the first known ubiquitin conjugate, monoubiquitinated histone H2A [4–8].

The discovery and biochemical characterization of protein A24 in 1973–1978 took place well before the human genome project, personal computers and the internet. In those days, protein characterization was slow, the sensitivity of detection was low, and concentrations of identified proteins in 2D gels were semiquantitative and nonlinear. All of this has changed now. The plethora of publications from models and clinical studies, and the extensive genomic, proteomic and publication databases, as well as advanced internet search tools, have greatly accelerated the identification of proteins, and have provided unprecedented access to the clinical and mechanistic implications of the proteomic

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changes identified in patients. The need to put all this information together has given birth to the new disciplines of bioinformatics, systems biology and pathway analysis. Matching proteomic results with available information from clinical and preclinical studies appears an overwhelmingly complex task. However, with the unbiased proteomics of blood, all of this is now achievable.

With appropriate quality controls in place, consistent and statistically significant differences in the concentration of certain groups of proteins in the blood of patients and age-matched normal and disease controls, can reflect meaningful indicators of disease processes. These indications are useful for patient diagnosis, differential diagnosis and monitoring, rising to the level of the principal of complementarity and the level of the whole organism, the human being and disease.

Why 2D gels?

2D gel electrophoresis has been used in research laboratories for biomarker discovery since the 1970s [1–12]. In the past, this method was considered highly specialized, labor-intensive and nonreproducible.

Now, however, the reproducibility of 2D gel technology has been markedly enhanced and its applicability expanded by advanced raw materials, equipment and software, and by biochemical processing, digital image analysis, biostatistical protocols and quality control. We are now applying these improved technologies from discovery through clinical validation, so that data can ultimately be suitable for regulatory submission [101].

For unbiased proteomics, 2D gel electrophoresis is the best approach. There are few comparable alternatives to 2D gel electrophoresis for tracking changes in protein expression patterns related to disease. 2D gel electrophoresis provides separation of unique intact protein species to detect changes in protein expression and to discriminate protein isoforms that arise due to variations in amino acid sequence and/or postsynthetic protein modifications, such as phosphorylation, ubiquitination, conjugation with ubiquitin-like proteins, acetylation, glycosylation and proteolytic processing. These are important variables in regulatory processes. Different isoforms, splice variants, post-translational modifications and precursor processing variations can indicate alternative disease processes are at work in the patient.

The protein biomarkers must be sufficiently characterized to avoid the pitfalls of protein isoform differences. It is critical to know which protein molecular entity is the actual biomarker. This is a major caveat that underscores the advantages of the level of intact protein fractionation that 2D gel electrophoresis can provide.

Our experience at Power3 indicates that quantitative 2D gel electrophoresis can provide the required quality of information. Repetitive analysis of protein spots over the dynamic range of the assay using standard blood serum samples clearly indicates that the analytical process is reproducible and robust (Figure 1 & Appendix 1). In 2006–2007, we published results of our initial application of the new paradigm, in which we interpreted an unbiased proteomic comparison by quantitative 2D gel electrophoresis of blood serum from patients with sporadic Lou Gehrig's disease (sporadic amyotrophic lateral sclerosis [sALS]), familial ALS (fALS), Parkinson's disease (PD), Alzheimer's disease (AD) and AD-like dementias [13–15]. I will use some of Power3 Medical's results to illustrate the concept that unbiased proteomic results from the blood serum of patients and control subjects relate to the results of preclinical and clinical studies. I will also try to uncover disease mechanisms as identified in patients, being measured and monitored by the proteomic results.

Using well-accepted multivariate biostatistical analysis methods with blood serum concentrations of groups of specific protein biomarkers, which we had identified as particularly useful, we consistently distinguished between patients with AD, ALS, PD and neurodegenerative diseases with similar symptoms to AD (AD-like). Clinical diagnostic quality sensitivities and specificities are obtained (Figure 2 & Appendix 2) [13–15].

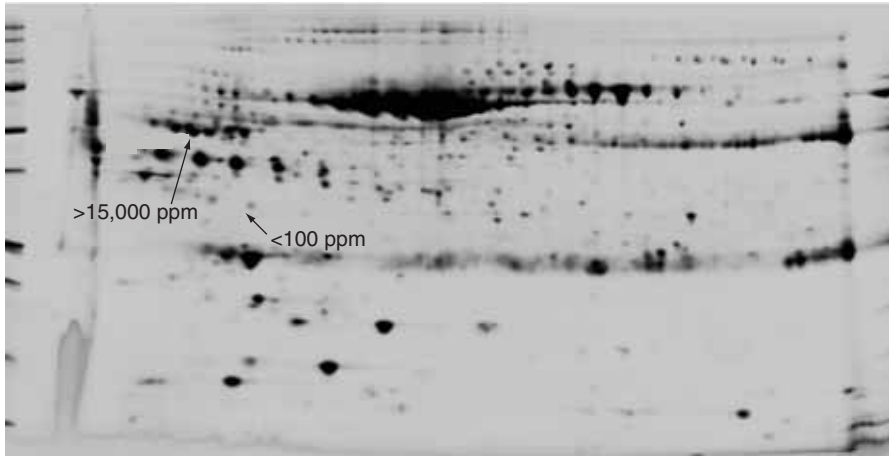

Figure 1. 2D gel of human blood serum. The dynamic range and reproducibility of quantitative 2D gel electrophoresis of human blood serum. More than 1500 protein spots are resolved and quantitated within the 2D gel pattern of human blood serum. Typical quantities, ranging from <100 to >15,000 ppm, are indicated to demonstrate the dynamic range (black arrows).

Shifting the paradigm: permission to connect the dots

Given this aggregate experience, when biomarker proteins are fully characterized, can one assert that in a patients blood, these quantitative protein differences reflect the disease processes that these proteins are known to be involved in? Our own experience at Power3 indicates that we can. The following examples

underscore the usefulness of the quantitative and qualitative information that 2D gel electrophoresis is designed to provide.

Biomarker N7: isoform-specific differences between Alzheimer's & Parkinson's diseases

It is particularly important to validate results of multivariate biostatistics with individual biomarker statistics in order to ensure that the multivariate results arise from statistically significant individual differences. Using well-accepted single-variable biostatistics, we also found consistent differences in blood serum concentrations of individual biomarkers, reflecting the utility of the biomarkers in multivariate analysis when they are combined (Figures 3 & 4, Appendices 2–5).

As illustrated in Figure 4 and Appendices 4 & 5, biomarker protein N7 is elevated in blood serum concentrations in AD above age-matched normal controls, whereas it is not changed in AD-like and mixed dementias, and is decreased in the blood serum concentration of PD patients.

On the other hand, the same PD patients' samples show no difference from age-matched controls in the blood serum concentration of another isoform of this same protein, an isoform that is also revealed by 2D gel electrophoresis.

	Summary statistics using 34 biomarkers			Summary statistics using 24 biomarkers		
Disease	AD (n = 22)	PD (n = 29)	ALS (n = 136)	AD (n = 44)	AD-like (n = 24)	Normal (n = 23)
Differential diagnosis (%)	91	79	89	86	92	94

Figure 2. Differential diagnostics through multivariate biostatistics of blood serum concentrations of groups of proteins using quantitative 2D gel electrophoresis. Disease-specific discrimination was accomplished using both canonical discriminant analysis (top) and linear discriminant analysis (bottom), showing discrimination between patients with AD, PD and ALS **(A)** and patients with AD, AD-like and normal controls **(B)**.
AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; PD: Parkinson's disease.
Data from [14,15].

Sporadic & familial ALS: different effects on biomarker phosphorylation isoforms N1 & N2

Two blood serum proteins, biomarkers N1 and N2, are separated by 2D gel electrophoresis in the isoelectric focusing first dimension, but not in the SDS second dimension [13,14].

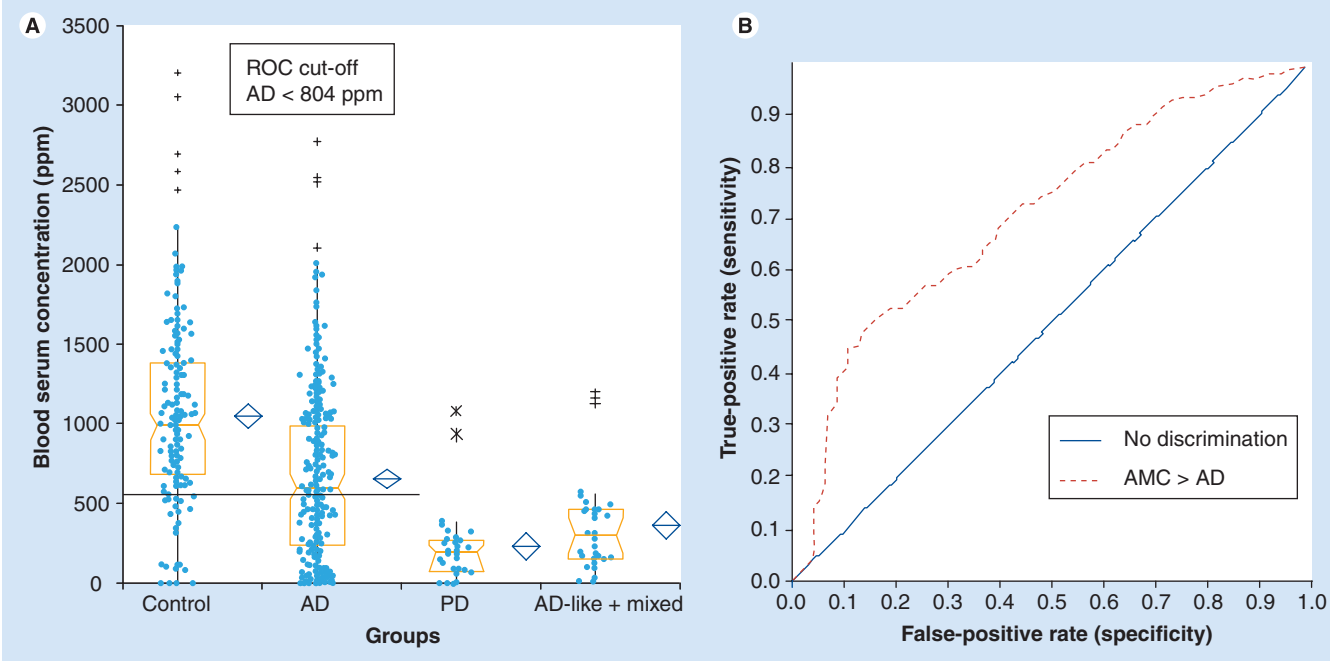

Figure 3. Statistical pattern of N3 serum protein biomarker in different neurodegenerative diseases. (A) Box and whiskers quantitative level and **(B)** capability of differentiation between AD and AMC.
AD: Alzheimer's disease; AMC: Age-matched controls; PD: Parkinson's disease; ROC: Receiver operator characteristics.

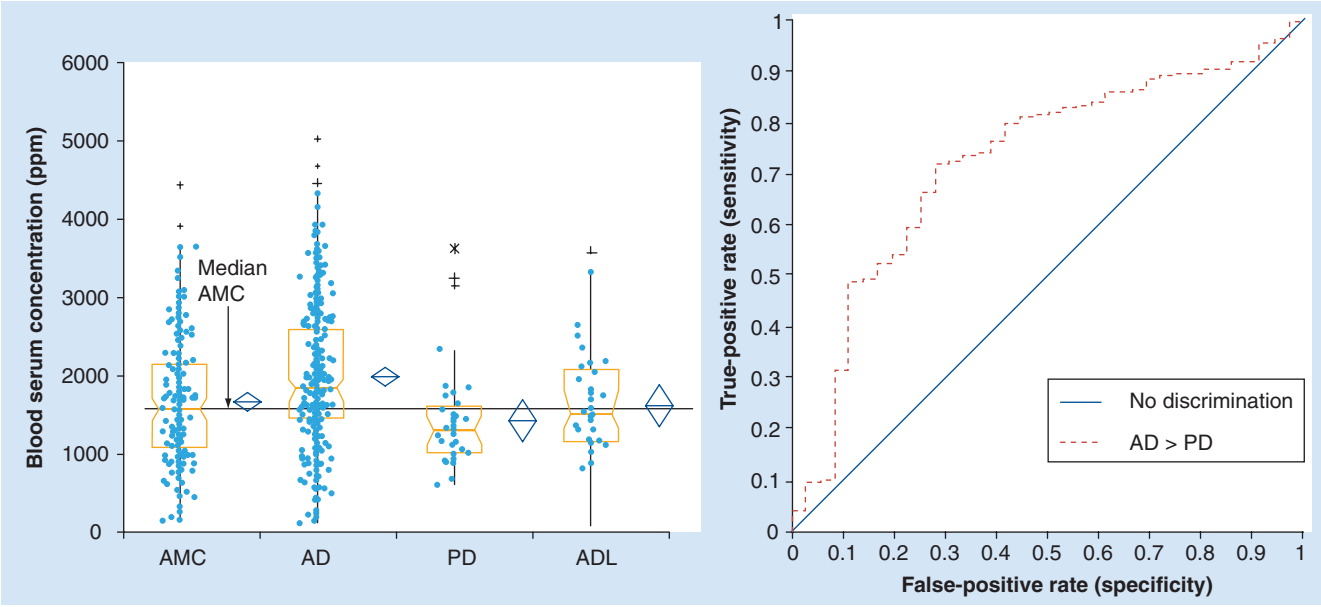

Figure 4. Statistical pattern of N7 serum protein biomarker in different neurodegenerative diseases. (A) Box and whiskers quantitative level and **(B)** capability of differentiation between AD and PD.
AD: Alzheimer's disease; ADL: AD-like and mixed; AMC: Age-matched controls; PD: Parkinson's disease.

Biomarkers N1 and N2 have the same amino acid sequence. The N1 protein is tyrosine phosphorylated whereas N2 is not [13]. Based upon what is known about the mechanisms these proteins are involved in [16–26], elevation of N1 phosphoprotein concentration in blood serum indicates increased autoimmune inflammation, whereas elevation of N2 protein blood serum concentration indicates increased innate inflammation [13–15].

Box 1. Benefits of blood serum proteomics.

The examples illustrate how blood serum proteomics:

- Provides a relational perspective from the patients to functional, preclinical and clinical studies of genomic and proteomic biomarkers.
- Enables differential diagnostic and disease-specific mechanism discrimination between:
  - Similar diseases (e.g., Alzheimer's disease vs Lou Gehrig's disease vs Parkinson's disease; Alzheimer's disease vs Alzheimer's disease-like vs Parkinson's disease, vs age-matched normal)
  - Sporadic and familial disease subcategories (e.g., sporadic Lou Gehrig's disease vs familial Lou Gehrig's disease)
  - Disease mechanisms (e.g., oxidative stress, apoptosis, and autoimmune and innate inflammatory mechanisms of neuronal degeneration)
- Provides the type of information that can be employed in the monitoring of patients for:
  - Potential drug response
  - Disease severity and progression
  - Potential new drug targets
- Will ultimately lead to personalized medicine.

In sALS, the N1 phosphoprotein is elevated in blood serum concentration over age-matched normal controls, whereas the N2 protein is decreased [13,14]. In fALS, the N1 protein is also elevated in blood serum (to a lesser extent than in sALS) and an equal elevation of N2 in fALS is also noted [13,14]. These differences make perfect sense because ALS patient serum samples contain antimotor neuron antibodies [27–29] and fALS is caused by mutant mitochondrial proteins (e.g., superoxide dismutase) that induce intraneuronal cell mitochondrial leakage, leading to intracellular oxidative stress, apoptosis and inflammation (Appendix 7) [30–32]. Similar considerations regadring these two proteins along with the quantitative pattern of other biomarkers help distinguish between AD versus PD versus AD-like and PD-like diseases (Appendices 1–6) [14,15,33–36].

Summary remarks

Unbiased proteomics of blood serum can tell us what disease pathways and mechanisms of neuronal degeneration are active in the patients. For single protein biomarkers, we have illustrated statistically significant differences in blood-serum concentrations between patients with AD, ALS, PD, AD-like diseases and age-matched normal controls, as well as differences between familial and sALS. The theme is neurodegeneration and the variations are the different mechanisms through which it is acheived: oxidative stress, apoptosis, autoimmune, acquired and innate inflammation, and others. The blood reflects all this and more. Furthermore, employing multiple protein biomarker combinations, and with additional blood serum proteins brought into the analysis, we have also demonstrated that the disease-specific

differences have joint capabilities for sensitive and specific differential diagnosis, implying that disease-specific mechanistic differences measured by blood serum proteomics will ultimately lead to differential treatment and personalized medicine (Box 1).

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Financial & competing interests disclosure

The author is an officer and director of Power3 Medical Products, Inc as disclosed in the Company's filings with the US Securities and Exchange Commission. He is also an inventor of several patents pending assigned to the company. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Appendices

- Appendix 1: Reproducible quantitation of 13 different protein spots.
- Appendix 2: Mean ± standard error of N3 in different neurodegenerative diseases and statistical comparison to age-matched control.
- Appendix 3: Receiver operator characteristics AUC and cut-off value that provides maximum sensitivity and specificity for N3 when used as a single biomarker to differentiate between Alzheimer's disease and age-matched control.
- Appendix 4: Mean (ppm) ± standard error of N7 in different neurodegenerative diseases and statistical comparison to age-matched control.
- Appendix 5: Receiver operator characteristics AUC and cut-off values that provide maximum sensitivity and specificity for N7 when used as a single biomarker to differentiate AD from PD, AD-like or age-matched control.
- Appendix 6: Mechanism of neuronal degeneration measured in blood.
- Appendix 7: Disease processes measured in blood of ALS patients.

Appendix 1. Reproducible quantitation of 13 different protein spots.

Biomarker	Number	Mean ± standard deviation error	Coefficient of variation ≤20%
M1	14	13542	711
M2	14	3853	140
M3	14	1413	52
M4	14	1015	49
M5	14	678	28
M6	14	655	33
M7	14	595	31
M8	14	469	26
M9	14	359	16
M10	14	209	11
M11	14	129	5
M12	14	106	6
M13	14	72	4

Illustrates the reproducibility of quantitation of protein spots over the dynamic range of the 2D gel assay of human serum depicted in **Figure 1**. Reproducible quantitation of 13 different protein spots covering the dynamic range was obtained by running the same sample of human blood serum on 14 different 2D gels. The gels were run by different technicians on different days over 2.5 months (n = 14; range 72–13,542 ppm). The reproducibility was demonstrated with a coefficient of variation of ≤20% (n = 14), independent of the protein concentration, where the lowest concentration spot, 72 ppm, is approximately tenfold higher than the limit of detection (100 pg/spot = ~5–10 ppm) of the assay.

Appendix 2. Mean ± standard error of N3 in different neurodegenerative diseases and statistical comparison to age-matched control.

N3	Number	Mean (ppm)	± standard error	AMC (%)	ANOVA-P
AMC	75	1047.7	36.88	100	< 0.0001
Alzheimer's disease	115	656.0	27.01	63	
Parkinson's disease	12	230.8	42.58	22	
Alzheimer's disease-like plus mixed	12	355.4	48.89	34	

AMC: Age-matched control; ANOVA-P: p-value of analysis of variance.

Appendix 3. Receiver operator characteristics AUC and cut-off value that provides maximum sensitivity and specificity for N3 when used as a single biomarker to differentiate between Alzheimer's disease and age-matched control.

N3 (Alzheimer's disease < AMC)	ROC (N3 < cut-off)	Sensitivity	Specificity	Area	SE	ROC-P
AMC			64.0%	0.71	0.022	<0.0001
Alzheimer's disease	804	64.1%				

See (**Figure 3**).
AMC: Age-matched control; ROC: Receiver operator characteristics; ROC-P: p-value of ROC; SE: Standard error.

Appendix 4. Mean (ppm) ± standard error of N7 in different neurodegenerative diseases and statistical comparison to age-matched control.

N7	Number	Mean (ppm)	± standard error	AMC (%)	ANOVA-P
AMC	75	1675	52.20	100	
AD	115	2000	48.48	119	<0.0001 AD > AMC
PD	12	1439	119.85	86	<0.0001 AD > PD
AD-like plus mixed	12	1624	138.90	97	<0.0001 AD > ADL

AD: Alzheimer's disease; AMC: Age-matched controls; ANOVA-P: p-value of analysis of variance; PD: Parkinson's disease.

Appendix 5. Receiver operator characteristics AUC and cut-off values that provide maximum sensitivity and specificity for N7 when used as a single biomarker to differentiate AD from PD, AD-like or age-matched control.*

N7 (AD < AMC)	ROC (N7 < cut-off)	Sensitivity (%)	Specificity (%)	Area	Standard error	ROC-P
AD > AMC	1698	56.2	56.4	0.61	0.024	<0.0001
AD > PD	1500	71.9	72.2	0.72	0.0244	<0.0001
AD > ADL	1693	56.8	58.3	0.62	0.049	<0.007

*Best performance for N7 when used to differentiate between AD and PD.
See (FIGURE 4).
AD: Alzheimer's disease; ADL: Alzheimer's disease-like; AMC: Age-matched controls; PD: Parkinson's disease; ROC-P: p-value of receiver operator characteristics; ROC-P: p-value of ROC.

Appendix 6. Mechanism of neuronal degeneration measured in blood.

Mechanisms	AD-like mixed dementias	Parkinson's disease	Alzheimer's disease
N1: Autoimmune activity	Unchanged	Increased	Increased
N2: Innate inflammatory activity	Increased	Increased	Increased
N3: Inhibition of extracellular signals for apoptosis	Decreased	Decreased	Decreased
N4: Inhibition of escape from cell degeneration	Increased (stroke-related and mixed dementias only)	Increased	Increased
N5: Innate inflammatory activity	Increased (stroke-related and mixed dementias only)	Increased	Increased

Appendix 7. Disease processes measured in blood of ALS patients.*

Mechanism of neuronal degeneration	Amyotrophic lateral sclerosis	
	Familial	*Sporadic*
Neuronal oxidative stress and apoptosis	Intracellular primary	Inhibited pathway
Autoimmune, innate and/or acquired inflammation	Secondary innate and acquired	Primary autoimmune

*See [13,14].