A type 2 biomarker separates relapsing-remitting from secondary progressive multiple sclerosis

**ABSTRACT**

**Objective:** We tested whether it is possible to differentiate relapsing-remitting (RR) from secondary progressive (SP) disease stages in patients with multiple sclerosis (MS) using a combination of nuclear magnetic resonance (NMR) metabolomics and partial least squares discriminant analysis (PLS-DA) of biofluids, which makes no assumptions on the underlying mechanisms of disease.

**Methods:** Serum samples were obtained from patients with primary progressive MS (PPMS), SPMS, and RRMS; patients with other neurodegenerative conditions; and age-matched controls. Samples were analyzed by NMR and PLS-DA models were derived to separate disease groups.

**Results:** The PLS-DA models for serum samples from patients with MS enabled reliable differentiation between RRMS and SPMS. This approach also identified significant differences between the metabolite profiles of each of the MS groups (PP, SP, and RR) and the healthy controls, as well as predicting disease group membership with high specificity and sensitivity.

**Conclusions:** NMR metabolomics analysis of serum is a sensitive and robust method for differentiating between different stages of MS, yielding diagnostic markers without a priori knowledge of disease pathogenesis. Critically, this study identified and validated a type II biomarker for the RR to SP transition in patients with MS. This approach may be of considerable benefit in categorizing patients for treatment and as an outcome measure in future clinical trials.

**Classification of evidence:** This study provides Class II evidence that serum metabolite profiles accurately distinguish patients with different subtypes and stages of MS. Neurology 2014;83:1492–1499

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**GLOSSARY**

AD = Alzheimer disease; ALS = amyotrophic lateral sclerosis; AUC = area under the curve; MS = multiple sclerosis; NMR = nuclear magnetic resonance; PLS-DA = partial least squares discriminant analysis; PP = primary progressive; ROC = receiver operator characteristic; RR = relapsing-remitting; SP = secondary progressive.

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The transition from relapsing-remitting (RR) to secondary progressive (SP) multiple sclerosis (MS) occurs subtly and is difficult to define clinically. Identifying biomarkers that can distinguish between the different clinical phenotypes of MS is an important goal to ensure that the appropriate treatment regimens are adopted in a timely fashion. Furthermore, such biomarkers may provide new insight into the pathologic basis for the progressive process and lead to the development of effective treatments for disability prevention.

Metabolomic profiling of biofluids with high-resolution proton nuclear magnetic resonance (NMR) spectroscopy and partial least squares discriminant analysis (PLS-DA), a multivariate statistical pattern recognition technique, can be used to identify metabolites that vary in a correlated fashion within individual groups. This approach enables patterns of metabolite variation that are characteristic of a specific disease to be defined, rather than requiring identification of a unique, candidate-led biomarker. We have previously used this technique to show that animals with either predominantly macrophage-rich or neutrophil-rich brain pathologies...
can be differentiated from each other solely on the basis of their urine metabolite composition.\textsuperscript{10} Using a similar approach, characteristic metabolite patterns were identified in urine samples in a marmoset model of MS, as well as in samples from patients with MS compared with either healthy volunteers or other neurologic controls.\textsuperscript{11} These findings support the concept that biofluids analyzed by NMR/PLS-DA may be highly sensitive, not just to the presence of pathology within the brain, but to differences and changes in pathologic processes. In this study, we used NMR/PLS-DA analysis of serum metabolite composition to determine whether it is possible to objectively differentiate between MS clinical subtypes on the basis of metabolomic biofluid analysis.

**METHODS** Biofluid samples. We obtained serum samples from 3 independent cohorts (A, B, and C) of patients with MS (RR, SP, and primary progressive [PP]), as well as age- and sex-matched control volunteers, recruited from the John Radcliffe Hospital, Oxford, UK (see STARD flowchart of patient recruitment, exclusion, and methodology in figure 1). We diagnosed patients with MS using the gold standard 2001 and 2005 McDonald criteria, with at least 2 clinical attacks for RR phenotypes\textsuperscript{12,13} and continuous worsening of neurologic impairment over at least 12 months, not explained by incomplete recovery from relapses, for those defined as SPMS. Of the 58 patients with SPMS in the study, 53 did not have a relapse in the 2 years before and after sampling, 3 had one relapse, and data on the remaining 2 are not available. No patients with PPMS had any recorded relapses. A consultant neurologist performed the clinical assessments of all patients with MS (J.P.). Overall patient demographics are shown in table 1, and a more detailed breakdown together with a list of therapies is provided in table e-1 on the Neurology\textsuperscript{8} Web site at Neurology.org. Blood samples were collected and allowed to clot for 30 minutes at room temperature in Vacutainers containing clot-activator and gel (BD, UK). Samples were centrifuged (3,000 g, 10 minutes) and the serum aliquoted and stored at -80°C. Samples were excluded...
Table 1  Patient demographic information

<table>
<thead>
<tr>
<th></th>
<th>PPMS</th>
<th>RRMS</th>
<th>SPMS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (range) age, y</td>
<td>58 (43–63)a</td>
<td>40 (22–62)</td>
<td>51 (28–65)b</td>
<td>54 (18–75)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>6 (46)</td>
<td>15 (58)</td>
<td>43 (74)</td>
<td>16 (67)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>7 (54)</td>
<td>11 (42)</td>
<td>15 (28)</td>
<td>8 (33)</td>
</tr>
<tr>
<td>Median (IQR) EDSS</td>
<td>6 (4.4–6.4)</td>
<td>2 (1–5.4)</td>
<td>6 (5.75–6.5)c</td>
<td>0 (0–0)d</td>
</tr>
<tr>
<td>Median (range) TSR, mo</td>
<td>NA</td>
<td>20 (1–161)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Median (range) DD, mo</td>
<td>73 (17–333)</td>
<td>72 (6–318)</td>
<td>160 (65–430)</td>
<td>0 (0–0)d</td>
</tr>
</tbody>
</table>

Abbreviations: DD = disease duration; EDSS = Expanded Disability Status Scale; IQR = interquartile range; NA = not applicable; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; TSR = time since relapse.

More detailed breakdown of demographics and therapies received is given in Table e-1.

*p < 0.001 vs RRMS.

p < 0.05 vs RRMS.

*p < 0.001 with respect to RRMS.

p < 0.001 vs PPMS, RRMS, and SPMS.

from analysis if patients were found to have another major disease (e.g., diabetes mellitus [n = 1]), if the spectral quality was poor or grossly distorted (n = 15), or if the prepared sample was found to have a lipid content greater than 2 × SD from the mean of the group (n = 13, appendix e-1). For comparison to other neurologic diseases, serum was obtained from patients with either amyotrophic lateral sclerosis (ALS, n = 20, diagnosed by M.R.T.) or Alzheimer disease (AD, n = 10, diagnosed by G.K.W.) and compared with age-matched samples from healthy volunteers at the respective disease clinics (n = 14 and 20, respectively).

Standard protocol approvals, registrations, and patient consents. MS samples were collected and analyzed with ethical approval between 1995 and 2012 under project 08/H0604/155. ALS and AD patient samples were obtained from tissue banks and analyzed as part of the Oxford Study for Biomarkers in Motor Neurone Disease (BioMOrx, approved by the South Oxfordshire Research Ethics Committee 08/H0605/85) or the OPTIMA and Challenge cohorts with prior approval from the local research ethics committee (COREC 1656 and 96.243, respectively).

NMR spectroscopy. A 1D proton NMR spectrum was obtained for each sample using a Carr-Purcell-Meiboom-Gill pulse sequence as previously described, with some slight modifications. Prior to spectroscopy, samples were defrosted at 4°C, then further centrifuged (100,000 × g, 30 minutes, 4°C). Serum (150 μL) was mixed with 0.24 M sodium phosphate NMR buffer prepared in D2O (pH 7.4) to a final volume of 600 μL. Mixed samples were transferred to a 5-mm NMR tube and spectra acquired using a 700-MHz spectrometer operating at 16.4T (Bruker Avance III, Billerica, MA). One sample from each batch was analyzed by 2D spectroscopy to aid metabolite identification. More detailed NMR methods are provided in appendix e-1.

Data preprocessing. Spectra were imported into MATLAB (Mathworks, Natick, MA) and scaled using the internal standard to ensure correct amplitude. Further preprocessing corrected for subtle shifts in peak position arising from pH differences between samples. The remaining processing was carried out as previously described, with slight modifications. Briefly, the spectra were subdivided into 0.02-ppm buckets from 0.20 to 9.60 ppm (6 = midpoint of integral region). In each spectrum, buckets in the 4.30–5.00 ppm region, which is highly variable owing to imperfect water suppression, were excluded. In addition, buckets containing gross contaminants (e.g., organic solvent) were excluded from analysis. Each remaining bucket was integrated, yielding 420 independent variables for each sample. Prior to multivariate statistical analysis, data were scaled using the Pareto variance to suppress noise. More detailed methodology is available in appendix e-1.

Multivariate statistical analysis. For each comparison, we used SIMCA 13 (Umetrics, Sweden) to construct a PLS-DA model that best explained the differences between the variables for the groups being studied. Each model was assessed for predictivity by calculating the q2 value. A q2 > 0.4 is the threshold for significance in biological modeling. Models were further validated by a Monte Carlo method where 100 models were built using random group assignments. Only models where the genuine q2 was higher than 95% of the randomly generated q2 values were considered significant. More detailed statistical methods are included in appendix e-1.

Independent validation. Two sets of patients, A and B, were used to build independent predictive models. Each model’s loadings and contributions were compared to ensure similarity in variables driving separation. In addition, a third independent set of samples, C, was used as a testing set. A researcher blinded to all clinical information tested the ability of the Set B model to predict group membership of the unknown Set C samples. The results were expressed as a 2 × 2 contingency table and receiver operator characteristic (ROC) curves were constructed. Model membership probability thresholds derived from the model-building set were used to determine sensitivity and specificity in the prediction set (Set C).

RESULTS Initial MS sample set (Set A). Spectra were obtained from the Set A serum samples and processed as described above to give integral values for each
independent bucket. Example spectra from a patient with RRMS and from a patient with SPMS are shown in figure e-1. Models built with data from Set A serum samples showed a significant separation between RR and SP groups ($q^2 = 0.45$, figure 2A). Examination of the loadings revealed that fatty acids ($\delta_{x-y} = 0.88, 1.30, 5.35$), phosphocholine ($\delta_{x-y} = 3.23$), an $N$-acetyl species ($\delta_{x-y} = 2.03$), and glucose ($\delta_{x-y} = 3.25, 3.75, 3.91$) were decreased in SPMS with respect to RRMS, whereas other fatty acids and $\beta$-hydroxybutyrate ($\delta_{x-y} = 1.19$) were increased. For other intra-MS comparisons, the model comparing the PP and RR groups appeared to differentiate 2 clear groups (figure e-2). However, a small number of overlapping patients in each group resulted in a nonpredictive $q^2$ value ($-0.11$). The PP vs SP model also returned a nonpredictive $q^2$ value ($-0.02$), with marked overlap (figure e-2).

We subsequently investigated the separation of patients with MS from an age- and sex-matched control cohort. A model differentiating all patients with MS from the control cohort was significantly predictive ($q^2 = 0.41$). Furthermore, the comparisons between individual stages of MS and control groups were also significant (figure 2, B and C). All models were successfully validated using the cross-validation method described above. Interestingly, when comparing each of the individual stages of MS against the controls, 2 metabolites were consistent across the groups: glucose ($\delta_{x-y} = 3.25, 3.75, 3.91$) and phosphocholine ($\delta_{x-y} = 3.23$) were both reduced. Further examination of both the RR vs control and SP vs control model loadings showed lactate ($\delta_{x-y} = 1.32$), a broad singlet-like resonance tentatively assigned to $N$-acetyl species ($\delta_{x-y} = 2.03$, previously assigned as $N$-acetyl glycoproteins$^{10}$), and some fatty acids were decreased in the patients with RRMS or patients with SPMS relative to controls. A separate subset of fatty acids was found to be elevated in the patients with RRMS and patients with SPMS relative to controls. Detailed metabolite findings are presented in table e-2.

Independent MS sample set analysis (Sets B and C). Samples from independent Set B were used in the same manner as the samples from Set A and 3 new models were generated: ControlB vs RRMSB, ControlB vs SPMSB, and RRMSB vs SPMSB. In each case, the models from Set B were significantly predictive ($q^2 > 0.4$) and were all validated in the same manner as the models from Set A. (ControlB vs RRMSB, $q^2 = 0.62$; ControlB vs SPMSB, $q^2 = 0.77$; and RRMSB vs SPMSB, $q^2 = 0.48$.) Moreover, upon examination of the loadings, the same metabolites were found to be responsible for the separations observed between groups in this secondary dataset with the exception of glucose, which had a lower variable importance in separating patients with SPMS from patients with RRMS in Set B.

Each new model generated from Set B was tested for its predictive ability using Set C, the independent
dataset that had not been used in building models. All Set B models were both sensitive and specific at predicting group membership for patients: RRMSB vs SPMSB, sensitivity = 0.9 and specificity = 0.8; RRMSB or SPMSB vs ControlB, sensitivity and specificity = 1.0. Graphical representations of models and prediction results (figure 3, A–C), and contingency tables (figure 3, D–F), demonstrate sensitivity and specificity of the models (Fisher exact test, 2-tailed). ROC plots (figure 3G) yielded areas under the curve (AUC) significantly greater than 0.5, indicating good predictive power (control vs RRMS, AUC = 1.00, p = 0.0045; control vs SPMS, AUC = 1.00, p = 0.0006; SPMS vs RRMS, AUC = 0.94, p = 0.0071).

Figure 3 Partial least squares discriminant analysis models and receiver operator characteristic curves demonstrate the selectivity and specificity of the model separating relapsing-remitting and secondary progressive multiple sclerosis.
Comparison with other neurologic diseases. The control vs RRMS model (figure 2C) was compared with models separating ALS and AD from age-matched control samples (figure 4, A and B, respectively). Neither of the latter 2 models was predictive, with a strong overlap of groups in both cases.

Effect of therapeutic agents, age, and sex. No significant models could be generated when modeling patients within our study randomized to either active therapy or placebo arms of the Cupid trial (tetrahydrocannabinol, $q^2 = -0.08$) or BioMS trial (Dirucotide, MBP-8298, $q^2 = -0.16$). Thus, the presence of these therapeutic agents did not contribute to the separations observed. Full details of therapies received are given in table e-1. Furthermore, when considering age as a factor within the control population, a model between older (≥46) and younger volunteers (<46) was not predictive ($q^2 = -0.19$). The cutoff of 46 was chosen as it lay halfway between the mean ages of the RRMS and SPMS cohorts. Likewise, when the sex of patients was highlighted on existing models (e.g., the Set A SPMS vs RRMS model), no discernible patterns were evident, with both sexes being distributed evenly. Thus, none of these variables (presence of therapeutic agent, age, or sex) contributed to the significant separations observed in our MS models.

DISCUSSION Using PLS-DA metabolomics analysis of serum NMR spectra, it was possible to generate validated predictive models that distinguished RRMS from SPMS, as well as separating each of the MS patient groups (RRMS, SPMS, or PPMS) from healthy control cohorts. This is Class II evidence. The majority of serum metabolites that underlay the model separations were identified. The sensitivity of the models to specific metabolites varied between each of the MS groups when compared with the control cohort, and most notably between PPMS and either of the other 2 groups, indicating sensitivity to the different disease states. The findings in other diseases (ALS and AD) served as a negative control, and confirmed that the predictive models generated in the MS cohorts were based on disease-specific metabolite changes and did not reflect common neurodegenerative processes per se.

It is of particular interest that our serum models generated a positive and significant separation between RR and SP patient groups. This separation was highly robust, with 2 independent sample sets (A and B) producing equivalent predictive models and identifying the same underlying metabolites. The only difference in the metabolites identified was glucose, which had a lesser influence in driving the models derived from Set B than Set A. This difference is likely to have arisen from variations in the timing of sample collection in the clinic and the period after the last meal. Critically, models created from Set B accurately predicted group membership of samples from the third, independent set (Set C). These findings strongly support the concept that NMR/PLS-DA analysis of serum samples can reliably differentiate between the RR and SP stages of MS disease progression. Based on the positive outcome of these relatively small, cross-sectional studies, further larger cohort, multicenter longitudinal trials are warranted to assess the potential of this approach both as a means to monitor transitioning from RRMS to SPMS and as a surrogate marker of time to SP disease in treatment trials.

Our models were not able to separate the progressive groups of MS from each other. This supports the

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**Figure 4 Models comparing different neurologic diseases to their respective controls**

**A**

![Graph](image)

$q^2 = -0.21$

**B**

![Graph](image)

$q^2 = -0.21$

Models to probe separation between 2 different neurologic diseases and control volunteers from their respective disease clinics. (A) Patients with amyotrophic lateral sclerosis (ALS) (purple hexagons) and control volunteers (gray stars); model is nonpredictive ($q^2 = -0.21$). (B) Patients with Alzheimer disease (AD) (turquoise diamonds) and control volunteers (gray stars); model is nonpredictive ($q^2 = -0.21$).
view that a similar pathologic mechanism underlies these phases.33 However, the lack of a predictive model between the PP and RR patients appeared to be disrupted by just 4 patients (3 RR and 1 PP) lying in the incorrect model region, suggesting that the underlying pathologic mechanisms of RRMS and PPMS are distinct. Although it is easy to distinguish RRMS from PPMS in the clinical setting, understanding the underlying pathogenic basis for PP disease is important. Thus, these observations warrant investigation and validation in larger cohorts. Further, a study to assess the potential of this approach in predicting conversion of clinically isolated syndromes to RRMS would be valuable and might help identify those patients for whom early treatment would be beneficial.

Previous studies of biomarkers in neurologic diseases, including MS, have investigated either individual or small numbers of indicators.20–23 However, despite differences identified at a group level, predictive value for an individual has been low.24 Whereas it is possible to separate patients with MS from controls using techniques such as MRL,12,13,25 or using a metabolomics-based approach on CSF samples,26 to our knowledge no studies have been reported in which patients with RRMS and SPMS have been separated from each other on the basis of serum NMR. As an anecdotal example of the potential strength of this approach, one patient with RRMS was found persistently to lie in the SPMS portion of the model. As this patient had been categorized some years previously, her neurologist was consulted to obtain her current status. The sample was collected in 2008 and at follow-up 1 year later the patient reported progressing over that year. By 2010, there was no doubt that the patient was progressing. These findings suggest that the metabolomic profile of the patient’s serum in 2008 was an early indicator of entering the progressive stage of disease.

It is important to note that, owing to its class-based learning approach, PLS-DA analysis reduces the impact of underlying conditions such as therapeutic status or diet-based effects, since these are likely to be randomly distributed throughout our sample population. If any variance in a population does not explain the difference between the user-defined classes, it is considered less important. This was demonstrated in our data as some patients were on blinded clinical trials. The models could discriminate between individuals with RRMS or SPMS in a manner that was independent of treatment regimen, suggesting that the metabolic changes caused by the disease progression were more consistent than those associated with the metabolism of any drugs present. This advantage of the PLS-DA approach is essential if it is to be useful clinically, as it must be able to discriminate between people with the specific disease states despite these nonspecific population variations.

The PLS-DA models developed here could be used to mark the change from RRMS to SPMS in clinical studies and, thus, enable onset of secondary progression to be used as an outcome measure in therapeutic trials.27 This is likely to be a more important outcome than relapse activity and better than short-term (3–6 months) disability change, which includes relapse-associated disability as well as progression. Moreover, this approach may identify key metabolites involved in the pathogenesis underlying progression of MS28 and identify targets for the development of novel therapies.

AUTHOR CONTRIBUTIONS

A.M. Dickens: contributed to the design of the principal experiments, performed the sample preprocessing for the NMR experiments, assisted with running the NMR experiments, performed the multivariate analysis, contributed to writing and proofing of the manuscript. J.R. Larkin: contributed to the design of the principal experiments, performed the sample preprocessing for the NMR experiments, assisted with running the NMR experiments, performed the multivariate analysis, contributed to writing and proofing of the manuscript. J.L. Griffin: designed the NMR sequences used in this study and assisted with the multivariate analysis, contributed to writing and proofing of the manuscript. A. Cavey: collected the MS samples used in this study and clinical data required for the samples, contributed to writing and proofing of the manuscript. L. Matthews: collected the MS samples used in this study and clinical data required for the samples, contributed to writing and proofing of the manuscript. M.R. Turner: collected the ALS samples used in this study, contributed to writing and proofing of the manuscript. G.K. Wilcock: collected the Alzheimer samples used in this study, contributed to writing and proofing of the manuscript. B.G. Davis: assisted with the interpretation of the NMR spectra and multivariate analysis, contributed to writing and proofing of the manuscript. T.D.W. Claridge: ran the NMR experiments in this study and assisted with metabolite identification from the NMR spectra, contributed to writing and proofing of the manuscript. J. Palace: contributed to the design of the principal experiments, provided the samples from the patients with MS and aided interpretation of the results, contributed to writing and proofing of the manuscript. D.C. Anthony: contributed to the design of the principal experiments and interpretation of the results, contributed to writing and proofing of the manuscript. N.R. Sibson: contributed to the design of the principal experiments, supervised all work and helped with the analysis and interpretation of the results, contributed to writing and proofing of the manuscript.

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