Integration of magnetic resonance imaging and protein and metabolite CSF measurements to enable early diagnosis of secondary progressive multiple sclerosis

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Abstract

Molecular networks in neurological diseases are complex. Despite this fact, contemporary biomarkers are in most cases interpreted in isolation, leading to a significant loss of information and power. We present an analytical approach to scrutinize and combine information from biomarkers originating from multiple sources with the aim of discovering a condensed set of biomarkers that in combination could distinguish the progressive degenerative phenotype of multiple sclerosis (SPMS) from the relapsing-remitting phenotype (RRMS).

Methods: Clinical and magnetic resonance imaging (MRI) data were integrated with data from protein and metabolite measurements of cerebrospinal fluid, and a method was developed to sift through all the variables to establish a small set of highly informative measurements. This prospective study included 16 SPMS patients, 30 RRMS patients and 10 controls. Protein concentrations were quantitated with multiplexed fluorescent bead-based immunoassays and ELISA. The metabolome was recorded using liquid chromatography-mass spectrometry. Clinical follow-up data of the SPMS patients were used to assess disease progression and development of disability.

Results: Eleven variables were in combination able to distinguish SPMS from RRMS patients with high confidence superior to any single measurement. The identified variables consisted of three MRI variables: the size of the spinal cord and the third ventricle and the total number of T1 hypointense lesions; six proteins: galectin-9, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor alpha (TGF-α), tumor necrosis factor alpha (TNF-α), soluble CD40L (sCD40L) and platelet-derived growth factor AA (PDGF-AA); and two metabolites: 20β-dihydrocortisol (20β-DHF) and indolepyruvate.

The proteins myelin basic protein (MBP) and macrophage-derived chemokine (MDC), as well as the metabolites 20β-DHF and 5,6-dihydroxyprostaglandin F1α (5,6-DH-PGF1α), were identified as potential biomarkers of disability progression.

Conclusion: Our study demonstrates, in a limited but well-defined and data-rich cohort, the importance and value of combining multiple biomarkers to aid diagnostics and track disease progression.

Key words: data integration, multiple sclerosis, disease progression, metabolomics, biomarker
Introduction

Traditionally, a biomarker is viewed as a characteristic that is objectively measured (e.g., a biological substance) and may affect interpretation or prediction of the incidence or outcome of disease [1,2]. In contrast, the molecular events in e.g., neurological diseases, are often complex, emphasizing that no single marker or source of information alone can reflect the full pathology. Recent advances in modern technologies have enabled comprehensive measurements at molecular and cellular levels, utilizing different biological specimens, tissues and radiological assessments. Considering the complexity of pathological events, integrating information from multiple sources can therefore result in a more refined tool for diagnostic and prognostic purposes. This concept is known as multianalyte assays with algorithmic analyses (MAAAs). Yet, the use of algorithm-based analyses in the clinic is very limited. A recent successful example of an MAAA is the improved test for prostate cancer [3].

Multiple sclerosis (MS) is an immune-mediated neurological disease that exists in two distinct phenotypes. At onset, the most common is relapsing-remitting MS (RRMS) in which focal inflammation in the central nervous system (CNS) appears over time, giving rise to clinical relapses, followed by a complete or partial recovery. Eventually, most RRMS patients will develop a progressively degenerative phenotype (secondary progressive MS, SPMS) with neurodegeneration, brain atrophy and accumulation of disability. The onset of SPMS is detected by continuous disability accumulation, monitored through the expanded disability status scale (EDSS) score and the presence of gadolinium (Gd) -enhancing lesions on MRI. One of the SPMS patients was on a disease-modifying drug (DMD) treatment, while 15 RRMS patients were on a DMD. The demographics of the cohort are summarized in Table 1 and DMD details in Table S3.

Methods

Ethics approval

All participants provided written informed consent, and the study was approved by the Regional Ethical Board of Uppsala (DNr 2008/182). Subjects

The study cohort consisted of 56 subjects: 30 were diagnosed with RRMS, 16 with SPMS, and 10 were controls with other, non-inflammatory, neurological diseases (e.g., idiopathic intracranial hypertension or thunderclap headache). All MS patients met the revised McDonald’s criteria for MS diagnosis [11]. Seventeen of the RRMS and two of the SPMS patients were inflammatory active with presence of gadolinium (Gd)-enhancing lesions on MRI. One of the SPMS patients was on a disease-modifying drug (DMD) treatment, while 15 RRMS patients were on a DMD. The demographics of the cohort are summarized in Table 1 and DMD details in Table S3.

Table 1. Clinical and demographic data including follow-up data of the patients. Four of the RRMS patients had transitioned to SPMS, one of whom was deceased.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Controls</th>
<th>RRMS</th>
<th>SPMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>On treatment, n</td>
<td>0</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>Age, mean(±SD)</td>
<td>39±(±13.1)</td>
<td>39±(±10.6)</td>
<td>56±(±9.3)</td>
</tr>
<tr>
<td>Female/Male</td>
<td>6/4</td>
<td>21/9</td>
<td>10/6</td>
</tr>
<tr>
<td>EDDS, median(range)</td>
<td>n/a</td>
<td>2.0(0–7.5)</td>
<td>5.5(3.0–7.5)</td>
</tr>
<tr>
<td>Disease duration in months, mean(±SD)</td>
<td>n/a</td>
<td>115±103.8</td>
<td>281±128.3</td>
</tr>
<tr>
<td>Follow-up</td>
<td>RRMS</td>
<td>SPMS</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>27</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>ΔEDSS, median(range)</td>
<td>0.0(3.5–3.0)</td>
<td>1.5(0–4.0)</td>
<td></td>
</tr>
<tr>
<td>Time interval in months, mean(±SD)</td>
<td>68±15.4</td>
<td>55±18.6</td>
<td></td>
</tr>
<tr>
<td>Transitioned, n</td>
<td>4</td>
<td>n/a</td>
<td>1</td>
</tr>
<tr>
<td>Deceased, n</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

EDSS: expanded disability status scale; n/a: not applicable; RRMS: relapsing-remitting multiple sclerosis; SD: standard deviation; SPMS: secondary progressive multiple sclerosis.

All participants underwent a clinical examination with EDSS grading and lumbar puncture at inclusion. MRI was performed within a week of the lumbar puncture at 1.5 T using the same imager and imaging protocol in all examinations. Gadopentetate dimeglumine (Magnevist®, 0.4 mL/kg body weight,
i.e., double dose) was used as a contrast agent, and MR images were analyzed visually. More details on how the T2 score, number of T1 and large T1 lesions were extracted can be found in the supplementary information, whereas other MRI-extracted measures have been previously published by us [12].

**Follow-up**

Baseline data were acquired for the 46 patients and 10 controls. Follow-up data were available for 40 patients. The mean follow-up time was 5±1.4 years. At the end of the follow-up, four patients had transitioned from RRMS to SPMS. One of these was deceased. These four patients were excluded from subsequent analyses and were used instead to evaluate the ability to predict SPMS at an early stage. The patients lacking follow-up data were assumed to have the diagnosis they were given at inclusion.

**Sample collection**

The lumbar puncture was performed through the L3/L4 or L4/L5 interspace, and CSF was collected in a polypropylene tube that was centrifuged for 5 min at 250 × g at room temperature. The supernatant was transferred, gently mixed and aliquoted in polypropylene tubes stored at -80 °C until analysis.

**Protein quantification**

CD27 was measured by sandwich ELISA kit, (DY382, R&D Systems, Minneapolis, MN, USA). The assay was calibrated against recombinant human CD27 and had a total coefficient of variation of approximately 5%.

CSF concentrations of amyloid β peptides ending at amino acids 38, 40 and 42 (Aβ38, Aβ40 and Aβ42, respectively) were measured using the Meso Scale Discovery Abeta Triplex assay (MSD, Rockville, MD). CSF T-tau and P-tau concentrations were measured using INNOTEST ELISAs (Fujirebio, Ghent, Belgium). CSF monocyte chemoattractant protein 1 (MCP-1) concentration was measured using the V-PLEX Human MCP-1 Kit (MSD, Rockville, MD). Intra-assay coefficients of variation were below 10% for all analyses.

Albumin concentrations in plasma and CSF were measured immunochemically in a Cobas 501 analyzer (Roche Diagnostics, Rotkreuz, Switzerland) with reagents from the same manufacturer. Experienced and board-certified laboratory technicians, blinded to clinical data, performed all analyses in one round of experiments using one batch of reagents per assay.

The remaining 39 out of the 52 clinical, radiological and protein (CRP) measurements had been previously characterized and published by us, including age and gender [12–15]. The mean and standard deviation of all CRP measurements in each diagnostic group are summarized in Table S4.

**Metabolite extraction**

CSF samples were thawed on ice, and 100 µL was transferred and mixed with 410 µL ice-cold methanol (MeOH) supplemented with an internal standard cocktail (D4-6keto-prostaglandin-F1-alpha, D4-thromboxane-B2, D4-prostaglandin-F2-alpha, D4-prostaglandin-E2, D4-prostaglandin-D2, D4-15-deoxy-delta12, 14-prostaglandin-J2, D4-cortisol and levonorgestrel) at a final concentration of 0.25 µM. The samples were further vortexed for 15 s and incubated at -20 °C for 30 min, followed by centrifugation at 20400 × g for 12 min at 4 °C. The supernatants were transferred to fresh Eppendorf tubes and dried using a centrifugal vacuum concentrator (overnight).

Upon analysis, the dried samples were reconstituted in 100 µL of 5% MeOH, 0.1% formic acid (FA) and 94.9% deionized MilliQ water. 10 µL of each sample was pooled to create a quality control (QC) sample to be injected repeatedly to monitor the performance throughout the analysis.

**Mass spectrometry analysis**

The samples were injected in a constrained randomized order, where they were divided into groups of eight that were injected twice for technical duplicates (the second injections were done in reversed order to prohibit patterns in potential carryover effects). A QC injection was done every 16th sample and a blank injection every 8th. Finally, a 2-fold serial dilution series ranging from 0.5 to 32 µL QC was injected.

The mass spectrometry analyses were performed using a Thermo Ultimate 3000 HPLC and Thermo Q-Exactive Orbitrap mass spectrometer. 10 µL sample was injected to a Thermo Accucore aQ RP C18 column (100 × 2.1 mm, 2.6 µm particle size). The analytical gradient was initiated with an isocratic flow for 3 min (0% B), followed by a 2.6 min gradient (0-10% B), 8.3 min (10-100% B) and 3 min (100% B), followed, finally, by re-equilibration and washing of the column for 3 min (0% B), where A is 0.1% FA in MilliQ water and B is 89.9% acetonitrile, 10% isopropanol and 0.1% FA. Mass spectrometry data were acquired in profile mode (in positive and negative ionization mode), using a mass range of 70–900 m/z during the first 5 min and 148–900 m/z in the following 15 min (to avoid low mass contaminants) in the positive ionization mode and 70–900 m/z throughout in negative ionization mode. To improve the identification of metabolites, eight tandem mass spectrometry analyses in positive and negative
ionization mode were performed separately on pooled samples (RRMS, SPMS, control and global pools).

Quantification

The acquired raw data was converted to an open source format (.mzML). Peaks were picked using msconvert from ProteoWizard [16] and preprocessed using the following pipeline within the OpenMS platform [17]. The peak-picked data were quantified by FeatureFinderMetabo [20], employing the Phenomenal infrastructure [21] and 2) the experimental MS2 spectra were manually curated and matched in mzcloud [22]. While mzcloud, in contrast to the de novo approach, has the advantage of matching against actual measured data on libraries of metabolites, it obviously lacks many metabolites that have not yet been measured and characterized, a weakness that is met by the de novo approach, where theoretical MS2 spectra for all metabolites in HMDB are present.

In cases when different identities were suggested by the two different approaches, the identity suggested by the de novo approach was chosen (implying that the identity was not present in mzcloud or that there was no clear match in mzcloud).

Metabolite identification

Two strategies were used for metabolite identification: 1) the experimentally measured peaks were compared to in silico fragmented spectra of the metabolites in the Human Metabolome Database (HMDB) [20], employing the Phenomenal infrastructure [21] and 2) the experimental MS2 spectra were manually curated and matched in mzcloud [22]. While mzcloud, in contrast to the de novo approach, has the advantage of matching against actual measured data on libraries of metabolites, it obviously lacks many metabolites that have not yet been measured and characterized, a weakness that is met by the de novo approach, where theoretical MS2 spectra for all metabolites in HMDB are present.

In cases when different identities were suggested by the two different approaches, the identity suggested by the de novo approach was chosen (implying that the identity was not present in mzcloud or that there was no clear match in mzcloud).

Statistical analysis

The quantified data was loaded into the statistical software environment R v3.4.0 [23] and features without established charge were removed. Blank filtering was performed according to our previously introduced pipeline [24]. A quality-filtering procedure was done using the dilution series requiring the spectral features in the series to have at least an absolute Pearson correlation of 0.5 with the injection volume. To stabilize variances, the intensity values were replaced by their log2 value, before potential sample outliers were investigated visually based on the total ion count of each sample. No sample outliers were found.

To reduce the intensity decrease over run time, the spectral features were normalized by the internal standards. The in-between-replicate Pearson correlation was calculated (minimum replicate correlation achieved was 0.95) and the replicates were averaged. Spearman’s rank correlation coefficients were calculated between the albumin ratio and the spectral features to eliminate features that may originate from blood (leaking through the blood-brain barrier). Features that acquired a statistically significant (p-value<0.05) absolute correlation higher than 0.5 were removed.

Finally, only metabolites with an established de novo identity were used in analysis with a coverage within metabolic features of at least 75%. Remaining missing values were replaced by the average feature value.

The age dependence was evaluated per metabolite and protein, assuming a linear relation using Pearson’s correlation analysis, where a p<0.05 was seen as a significant dependence. Age-dependent metabolites or proteins were age corrected by fitting a linear model (R function lm) to their levels in the controls and RRMS patients, with age as the explanatory variable. The age coefficient was extracted from the model and used to correct the metabolite or protein values in all individuals [25,26].

Multivariate modelling and variable selection

The CRP data and metabolomics data were separately subjected to partial least square discriminant analysis (PLS-DA) to target the differences between classes using the R package ropls [27], with the number of components predefined as two. This was performed using the class labels RRMS, SPMS and controls, comparing all groups against each other (referred to as the Global model) and RRMS vs. SPMS. The most significant variables were obtained using a combined “Variable Importance in the Projection” (VIP) which is commonly used to assess the importance of the X-variables in multivariate models.

To attain measures separating SPMS from RRMS that also differ from control levels, ten variables achieving the highest VIP values were extracted from each model, and the intersections between the Global and RRMS vs. SPMS variables were obtained. These variables (CRP and metabolic) were evaluated by PLS-DA separately, as reduced sets, and combined into a new dataset (CRPM), employing the same class labels as used previously (Global and RRMS vs. SPMS).

To reduce the risk of overfitting, i.e., to extract variation unique to the dataset alone, the full procedure, including the variable selection, was performed and evaluated using a 5-fold cross-validation (CV) repeated ten times. Briefly, the 5-fold CV divides the data into five balanced groups
using stratified sampling. Four of these groups are used for training the model, while the fifth is held out and used for validation and performance estimation. The procedure is repeated five times, so that each group may act as the validation cohort. Balanced error rates (BER), accounting for class sizes, were calculated for each model to estimate an overall predictive performance, and SPMS-specific error rates (ER) were obtained to establish the performance of SPMS detection. This analytical strategy is illustrated in Figure 1, and the complete code has been made publicly available on Github (https://github.com/stephanieherman/variable-selection). Additionally, to evaluate any potential overfitting, PLS-DA was performed on the CRP and metabolomics dataset including all individuals, and R2 and Q2 values were extracted.

The consensus variables (the ones with the highest average VIPs) were filtered for redundancies using Spearman’s ranked correlation analyses, excluding variables that were statistically significantly (p<0.05) correlated (r>0.80) as well as metabolites missing highly confident identities. Finally, to combine variable information, principal component analysis (PCA) was used (R function prcomp with centered and scaled variables) to compress the variables into two principal components. A Welch’s t-test was done between the RRMS and SPMS scores in the first principal component and for each separate variable alone. Similarly, the receiver operating characteristic (ROC) curves for each separate variable as well as the first principal component were computed with corresponding areas under the ROC curve (AUROC) using the R package pROC [28].

**Disease progression**

To identify variables that associate with the disease progression of SPMS patients, the increases in EDSS scores at the end of the follow-up period were assessed by an experienced neurologist and categorized into three groups by clinical degree of change. Group 1 consisted of patients whose EDSS scores were stable with no change during the follow-up period. An intermediate group 2 increased in EDSS by 0.5–1.5 if the initial EDSS was <5.5 or by 0.5–1 if the initial EDSS was ≥5.5. Finally, group 3 consisted of patients who had deteriorated significantly in the follow-up period, whose EDSS increased by >1.5 if the initial EDSS was <5.5 or by >1 if the initial EDSS was ≥5.5. This stratification aimed to take the clinical severity into account, which is not reflected by the EDSS scoring. The EDSS increase and corresponding group for each SPMS patient can be found in Table S6. To find variables that statistically significantly (p<0.05) varied between the groups, Kruskal-Wallis tests were done on the metabolites in the reduced metabolomics dataset as well as the CRP variables.

**Results**

**The CRP data distinguished RRMS and SPMS with a 22% error rate**

To investigate the ability to distinguish all groups (Global), the CRP data were modelled using PLS-DA after age correcting three age-dependent
proteins. A BER value for classifying all classes and an SPMS-specific ER were estimated (BER: 0.44±0.122, SPMS ER: 0.18±0.207), i.e., the SPMS was the only group that the model was able to moderately separate. To explore the variable contribution, VIP values were extracted, which indicated that EDSS contributed the most, followed by disease duration, age and the size of the spinal cord and third ventricle. EDSS, age and disease duration are essentially demonstrating that SPMS patients in general are older, have had the disease for a longer time and are more disabled than the other groups. These variables cannot be used for diagnostic purposes and were therefore excluded from further analyses. In addition, redundant variables giving the same type of information were also excluded from the CRP data (specifically large T1, T1 and T2 scores).

When these variables were removed, the performance was decreased (BER: 0.48±0.111, SPMS ER: 0.28±0.271). Excluding the control group, comparing RRMS and SPMS patients (RRMS vs. SPMS) resulted in a BER of 0.22±0.103 (Table 2). A visualization of the CRP RRMS vs. SPMS model built on all patients is shown in Figure 2A, where patients transitioning from RRMS to SPMS (n=4) were projected into the model space, with one of them located in the SPMS space and one in-between. Reducing the CRP variables down to the overlapping top ten variables from the Global and the RRMS vs. SPMS models resulted in improved performances for the Global model (BER: 0.38±0.135, SPMS ER: 0.23±0.249) and the RRMS vs. SPMS model (BER: 0.20±0.138).

### Table 2. The overall and SPMS-specific cross-validated (CV) error rates (ER) achieved by the respective models.

The overall CV error is a balanced error rate (BER), which is adjusted for class sizes so that 0.50 corresponds to random chance. Both the metabolomics and CRP models did not perform well for distinguishing between all groups (BER values close to 0.5). However, both models were moderately able to distinguish the SPMS patients from remaining groups. As for RRMS vs. SPMS, the CRP model obtained a BER value of 0.22 and the metabolomics model a BER value of 0.20. When reducing the variables to the overlapping top ten variables, the BER value was improved for the CRP model whereas it increased for the metabolomics model (0.20 and 0.30 respectively). Combining the top CRP and metabolic variables (CRPM) resulted in a BER value of 0.23, comparable to the value achieved by the full models.

<table>
<thead>
<tr>
<th>CV error, mean (±SD)</th>
<th>CRP model n=46</th>
<th>Metabolomics model n=606</th>
<th>Reduced CRP model n=1-10</th>
<th>Reduced metabolomics model n=1-10</th>
<th>CRPM model n=2-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (BER)</td>
<td>0.48±0.111</td>
<td>0.42±0.119</td>
<td>0.38±0.135</td>
<td>0.55±0.120</td>
<td>0.45±0.145</td>
</tr>
<tr>
<td>SPMS (ER)</td>
<td>0.28±0.271</td>
<td>0.26±0.248</td>
<td>0.23±0.249</td>
<td>0.39±0.302</td>
<td>0.34±0.230</td>
</tr>
<tr>
<td>RRMS vs. SPMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (BER)</td>
<td>0.22±0.103</td>
<td>0.20±0.142</td>
<td>0.20±0.138</td>
<td>0.30±0.167</td>
<td>0.23±0.149</td>
</tr>
</tbody>
</table>


**Figure 2. The CRP and metabolomics RRMS vs. SPMS models built using all measurements and patients.** The four transitioning patients have been projected into the model space and marked with blue stars. (A) A visualization of the CRP model with quality metrics of R²=0.70: p<0.05 and Q²=0.35: p<0.05. (B) A visualization of the metabolomics model with quality metrics of R²=0.87: p<0.05 and Q²=0.64: p<0.05. RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis.
The metabolome distinguished RRMS and SPMS with a 20% error rate

To attain the metabolomics signatures for further assessment, the CSF metabolomes of the study cohort were measured using liquid chromatography-mass spectrometry. After removal of contaminants and spectral features potentially originating from blood, a total of 606 metabolic features with a coverage of 75% (of which 21% correlated and were corrected for age) were used to build the metabolomics models. The Global metabolomics model (BER: 0.42±0.119, SPMS ER: 0.26±0.248) as well as the RRMS vs. SPMS model (BER of 0.20±0.142) attained higher performances than the CRP models, where the ERs again indicate that SPMS was the only group that the models were able to separate (Table 2). A visualization of the metabolomics RRMS vs. SPMS model built on all patients is shown in Figure 2B, where patients transitioning from RRMS to SPMS have been projected into the model space. Reducing the metabolites down to the overlapping top ten variables from the Global and the RRMS vs. SPMS models resulted in decreased performances for both models (Table 2).

Integration of CRP and metabolite measurements

To evaluate if a combination of CRP and metabolic variables can improve the prediction accuracy using only a limited number of variables, the overlapping top ten variables from the Global and RRMS vs. SPMS models of each dataset were combined into the CRPM dataset. Evaluated through CV over the full procedure (Figure 1), the CRPM Global model attained a comparable estimated performance (BER: 0.45±0.145, SPMS ER: 0.34±0.230), and the RRMS vs. SPMS model achieved a BER value of 0.23±0.149, which is comparable to that of the full models (Table 2).

The consensus of the overlapping top ten CRP variables from the Global and RRMS vs. SPMS models were: the size of the spinal cord and third ventricle, galectin-9, total T1, MCP-1, transforming growth factor alpha (TGF-α), tumor necrosis factor alpha (TNF-α), soluble CD40L (sCD40L) and platelet-derived growth factor AA (PDGF-AA). For the metabolomics data, the consensus overlapping top ten metabolic features were identified as: 5,6-dihydroxyprostaglandin F1a (5,6-DH-PGF1), 20β-dihydrocortisol (20β-DHF), indolepyruvate, and four that remained unidentified after manual curation, referred to as X1, X2, X3 and X4 (Table S7). These were combined into the consensus CRPM dataset that was filtered for potential redundancies in information using Spearman’s ranked correlation analyses between all variables. Metabolic features with an uncertain identity and variables that achieved a statistically significant (p<0.05) correlation higher

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**Figure 3. The Spearman’s ranked correlations between the consensus variables chosen in the variable selection.** Non-significant correlations (p>0.05) have been left blank.
than 0.80 were excluded (Figure 3). For correlating metabolites, the metabolite with the highest identification score from mzcloud was kept.

Finally, the CRPM dataset contained three MRI measurements, six proteins and two metabolites. Using these, the consensus CRPM RRMS vs. SPMS was compressed using PCA, now including all patients (except the transitioning patients), resulting in a large and highly significant (p=8.5×10^{-9}) separation over the first principal component (Figure 4A). Examining the variable loadings for the first principal component revealed that the size of the third ventricle, followed by galectin-9, contributed the most to the separation (Figure 4B). To investigate if the findings were therapy dependent, the PCA analysis was also performed excluding all patients on treatments (Figure S1), demonstrating an equally significant separation (p=8.1×10^{-9}), indicating that the findings are not explained by treatments.

In order to compare the integrated information to the separate variables alone, the separate variables were evaluated as the compressed information in the first principal component. Statistically significant differences were found between RRMS and SPMS for the size of the third ventricle: p=4.1×10^{-4}, galectin-9: p=0.007, total T1: p=0.008, 20β-DHF: p=0.002, MCP-1:
p=0.006, the size of the spinal cord: p=3.2×10^{-5}, indolepyruvate: p=0.002, TGF-α: p=0.01, sCD40L: p=0.007 and PDGF-AA: p=0.01, whereas TNF-α displayed a p=0.08 (Figure 4C).

In addition, the ROC with its corresponding AUROC value for the combined information in the first principal component was compared to the ROCs and AUROC values of the separate variables. An increase from 0.85 for the best performing variables (the size of the spinal cord and third ventricle) to 0.97 for the combined and compressed information was achieved (Figure 5), indicating that the combined information provided a higher performance.

**MBP, MDC, 20β-DHF and 5,6-DH-PGF1, associate to a worse disease progression in SPMS patients**

To identify CRP variables and metabolites that associate with the disease progression of SPMS patients, the levels of all CRP variables, including the metabolites in the reduced metabolomics dataset, were investigated using Kruskal-Wallis tests based on the three groups of varying degree of clinical change. The proteins myelin basic protein (MBP) (p=0.04) and macrophage-derived chemokine (MDC) (p=0.05) as well as the metabolites 20β-DHF (p=0.02) and 5,6-DH-PGF1 (p=0.05) achieved statistically significant differences between the groups (Figure 6). Interestingly, some of the transitioning patients (marked on the y-axis in Figure 6) displayed extreme levels of these proteins. Likewise, the combined information in the first principal component from the SPMS patients was investigated. No statistically significant difference (p=0.1) was found.

**Discussion**

We have shown that a model-based approach integrating clinical data, radiological, protein and metabolite measurements could be used for early diagnosis of SPMS patients. Using a linear combination of only eleven variables, the identification of SPMS patients could be improved. We also identified four biomarkers that were associated with a worse prognosis in patients with SPMS that could potentially be used to evaluate the disease course of these patients.
Multivariate diagnostics

Technological advances have enabled us to measure multiple layers of biological information. However, integrating the information into MAAs to be used in clinical care is still very rare. In health care, measurements are traditionally evaluated independently, ignoring any combinatorial effect provided by combinations of measurements. By taking advantage of multiple variables, logistic regression-based models have proven to be superior for screening in prostate cancer [3], and the result of regression of clinical data can assist in identifying patients thought to be at high risk of cardiovascular disease [29].

In support of our variable selection, most of the extracted variables have been previously associated with SPMS and/or have been linked to the neurodegenerative stages of MS, e.g., decrease in the size of the spinal cord [30,31], increase in the size of the third ventricle [32], and accumulation of the number of T1 lesions in the later stages of SPMS [33].

Others have demonstrated increased levels of galectin-9 in active MS lesions [34], and we previously demonstrated that galectin-9 was significantly elevated in SPMS patients in comparison to RRMS patients and healthy controls [14]. Here, we demonstrated that galectin-9 is the most valuable protein measurement for distinguishing SPMS patients and the second most important variable in the CRPM dataset. We and others have previously shown that MCP-1 (also termed CCL2) is elevated in SPMS patients compared to RRMS patients and that TNF-α is elevated in RRMS and SPMS patients compared to controls [15,35]. We have previously shown that sCD40L identifies RRMS patients with the presence of Gd lesions, whereas PDGF-AA decrease in SPMS patients compared to RRMS patients and controls [15]. Finally, the pro-inflammatory cytokine TGF-α has been shown to be associated to CNS lesions in MS [36]. However, none of these performed as well as the combined and compressed information for distinguishing RRMS and SPMS patients.

By defining three groups of varied clinical degree of impairment, we could demonstrate that MBP and MDC were elevated in SPMS patients with greater development of disability. MBP has been suggested as a potential marker for relapses in MS, as it is released during myelin sheath degradation [37,38], whereas MDC (also termed CCL22) is believed to play a role in various pathologies including the MS pathology and has been seen to be elevated in RRMS patients, in particular RRMS patients with Gd lesions, compared to controls [15] and to increase with disease progression in mice models with experimental autoimmune encephalomyelitis (EAE) [39]. CSF MDC has been found to be increased in female MS patients compared to male patients and female controls [40]. To investigate any potential gender effects on the MDC levels, Welch’s t-tests were done between all female and male patients, between female patients and controls and between female and male SPMS patients. We found no statistically significant difference between female and male patients (p=0.09), but a statistically significant increase was found in female patients compared to female controls (p=0.02). Amongst the SPMS patients, there was no statistically significant difference between female (n=10) and male (n=6) patients (p=0.8), indicating that the increase seen in the group of SPMS patients with the most severe disease progression is not due to gender. To our knowledge, the expression of MBP and MDC has not previously been demonstrated to associate with disease progression in SPMS patients.

A few metabolomics studies on CSF from MS patients have previously been performed, e.g., suggesting alterations in energy and phospholipid metabolism compared to non-MS [8,9], and MS plasma has shown dysregulation of the methionine metabolism, related to mitochondrial abnormalities [10]. Alterations in phospholipid metabolism have also been found in serum of SPMS patients with respect to RRMS patients [41]. The most discriminant metabolites found herein represent novel findings in MS to a large extent; 5,6-DH-PGF1 is a prostaglandin. Prostaglandin F2α has previously been shown to decrease in plasma and increase in CSF of progressive MS patients compared to other neurological diseases [42,43], suggesting that prostaglandins may be involved in the pathology of MS. Furthermore, indolepyruvate is involved in tryptophan metabolism, where it catalyzes the nonoxidative decarboxylation of 3-indolepyruvate to 3-indoleacetaldehyde. Indolepyruvate has also been found to act as a direct precursor for kynurenic acid in the presence of free radicals [44]. The kynurenine pathway is the major pathway in the tryptophan metabolism and has been recognized as central to the mechanisms of neurodegeneration [45]. The kynurenine pathway serum signature has also been shown to discriminate MS subtypes with an accuracy of 83% [46]. We found increased levels of indolepyruvate in SPMS patients compared to RRMS and controls that exhibited similar levels.

In additional support of our findings, both 20β-DHF and 5,6-DH-PGF1 were significantly increased in SPMS patients with greater development of disability, reinforcing their importance in the disease course of MS. Little is known about the glucocorticoid 20β-DHF, which is an abundant metabolite of cortisol. Previously, 20β-DHF measured
in urine has been associated with Cushing’s disease [47] and hypertension when measured in plasma [48]. Cortisol, however, has been reported to increase in the plasma and urine of MS patients [49–51]. Herein, we also find a statistically significant increase of CSF cortisol levels in SPMS patients compared to RRMS patients (Figure S2). However, we found the relative importance of SPMS diagnosis to be much higher for 20β-DHF than that of cortisol.

**Assessment of early diagnosis of SPMS patients**

In the study cohort, the clinical follow-up of patients showed that four patients had transitioned from RRMS to SPMS within two to three years. As SPMS currently is diagnosed retrospectively, when the neurodegeneration potentially has been present for years, there is a possibility that these patients already had a neurodegenerative phenotype at the start of the study. Therefore, these patients were kept out of the variable selection procedure and instead assessed for early detection of SPMS. In the first principal component of the CRPM variables, three transitioning patients and one RRMS patient were closer to the SPMS patients than the RRMS patients (one with a score above the 95% confidence interval of the average SPMS score; (CI: 1.15, 2.39), p=2.1×10⁻⁵), indicating that these patients were at high risk for development of SPMS. As for the remaining transitioning patient who displayed values closer to the RRMS patients, the patient had either not developed a neurodegenerative profile or the neurodegeneration was too mild to be detected by the measurements included in the current study.

**Methodological aspects and limitations of the study**

Our goal was to combine information of carefully chosen variables for early identification of SPMS patients. The methodological strategy employed herein is novel to our knowledge, but the concept of information integration as MAAAs has been under discussion and employed for some time [3,52,53].

Three MRI, six protein and two metabolite measurements constructed the final CRPM dataset. When these were compressed down to a linear combination using PCA, the first principal component showed an increase in AUROC from 0.85, which was yielded by the best-performing single measurements, to 0.97. To estimate the value of adding the two metabolites 20β-DHF and indolepyruvate, the predictive accuracy of the compressed information of the final CRP variables was calculated (AUROC=0.93), indicating a modest increase in accuracy when adding the two metabolites.

Random forest based on protein ratios has previously proven valuable for MS diagnostics. Using a set of 22/21 protein ratios, Barbour et al. achieved AUROC values of 0.98/0.91 (ERs of 0.09/0.11) when distinguishing MS from non-MS and progressive MS (SPMS and primary progressive MS, PPMS) from RRMS, validated on an independent cohort [7].

Using a multivariate approach like PLS-DA, measurements will be combined in an optimal way to maximize the variance among the groups of interest. If for example variable X and Y perform poorly independently, but very good when combined, they will be discarded by random forest but will be picked up by PLS-DA. However, the prevalent drawback of PLS-DA is the risk of overfitting, which we accounted for by performing repeated CV. The objective of CV is to extract true global variation, while ignoring variation unique to the dataset alone. An independent validation cohort as utilized by Barbour et al. would of course be superior to this approach and of great value to confirm our findings [7]. To check for overfitting in the models used in the variable selection, the models were rebuilt using all patients and the R² and Q² were extracted, where R² describes how well the data explain the groups and Q² is analogous to R² except for being based on CV. The CRP (n=46) RRMS vs. SPMS model resulted in R²=0.70, p<0.05 and Q²=0.35, p<0.05 and the metabolomics RRMS vs. SPMS model utilizing all metabolites (n=606) demonstrated values of R²=0.87, p<0.05 and Q²=0.64, p<0.05, indicating that the metabolomics model demonstrates higher retention of predictivity, i.e., less overfitting (R²-Q²), and a higher overall predictivity (Q² value). Similarly, but with lower values due to the inability to distinguish RRMS from controls, the Global models achieved values of R²=0.40, p<0.05 and Q²=0.16, p<0.05 (CRP) and R²=0.48, p<0.2 and Q²=0.28, p<0.05 (metabolomics). Essentially this indicates that the metabolomics and the CRP models that we use to extract the CRPM variables contain information that should be important in the global MS population.

To illustrate the final variables chosen by the variable selection included in CRPM, an unsupervised multivariate method was used (PCA). PCA works in a similar manner as PLS-DA, by decomposing the data into linear combinations of the variables. However, instead of maximizing the variation between predefined groups of interest, it maximizes present variation in the data not including any prior knowledge of grouping. PCA stores the most dominant variation in the first principal component
and decreasing amounts of variation in the subsequent components, meaning the first principal component contains the most information. The first principal component of the combined CRPM variables showed a highly significant separation between the RRMS and SPMS patients ($p=8.5\times10^{-9}$), superior to the best performing separate variable ($p=3.2\times10^{-5}$).

The most prominent limitation of the study is the limited number of patients and controls. While this increases the risk that our data do not accurately reflect the population, single-center studies have the advantage of avoiding inter-center variabilities and other confounders that can be difficult to correct for or handle [24]. Additionally, there were only four patients who transitioned from RRMS to SPMS during the follow-up period, limiting our findings for early detection. The fact that Barbour et al. attained a different set of protein markers (then evaluated as protein ratios) than extracted herein indicates that there are more proteins of interest for use in MS diagnostics [7]. Finally, as half of the RRMS patients ($n=15$) and one of the SPMS patients were on ongoing treatments, these treatments could potentially affect the top candidate variables. To assure that this was not the case, a PCA was performed on the CRPM variables, excluding all patients on ongoing treatments (Figure S1). The results showed that the separation between the RRMS and SPMS patients remained highly statistically significant over the first principal component ($p=8.1\times10^{-9}$).

**Future perspectives**

Integrative approaches hold great potential in future diagnostic assessments of many diseases, and as a general concept (MAAA) they could be applied to any medical condition with quantitative measurements. The challenges faced when translating this into routine health care would be the validation of a combinatorial scoring system, as the combined information as well as the single measurements included would need to be characterized.

We also identified proteins and metabolites that were associated with development of disability. This may prove to be important in identifying patients at high risk for development of disability and thus highly suited for clinical trials of treatments for progressive MS. Furthermore, whether the levels of these proteins and metabolites can be influenced by therapeutic intervention will be an important area for future research.

**Abbreviations**

20β-DHF: 20β-dihydrocortisol; 5,6-DH-PGF1: 5,6-dihydroxyprostaglandin F1α; AUROC: area under the receiver operating characteristics curve; BER: balanced error rate; CNS: central nervous system; CRP: clinical, radiological; CRPM: clinical, radiological, protein, metabolite; CSF: cerebrospinal fluid; CV: cross-validation; EDSS: expanded disability status scale; ER: error rate; FA: formic acid; Gd: gadolinium-enhancing; MAAA: multianalyte assays with algorithmic analyses; MBP: myelin basic protein; MCP-I: monocyte chemoattractant protein-1; MDC: macrophage-derived chemokine; MRI: magnetic resonance imaging; MS: multiple sclerosis; NS: non-significant; PCA: principal component analysis; PDGF-AA: platelet-derived growth factor AA; PLS-DA: partial least squares discriminant analysis; QC: quality control, protein; ROC: receiver operating characteristics; RRMS: relapsing-remitting multiple sclerosis; sCD40L: soluble CD40L; SPMS: secondary progressive multiple sclerosis; TGF-α: transforming growth factor alpha; TNF-α: tumor necrosis factor alpha; VIP: variable importance in the projection.


