Multiple sclerosis is an inflammatory, demyelinating, and neurodegenerative disease of the central nervous system. In most patients, the disease initiates with an episode of neurological disturbance referred to as clinically isolated syndrome, but not all patients with this syndrome develop multiple sclerosis over time, and currently, there is no clinical test that can conclusively establish whether a patient with a clinically isolated syndrome will eventually develop clinically defined multiple sclerosis. Here, we took advantage of the capabilities of targeted mass spectrometry to establish a diagnostic molecular classifier with high sensitivity and specificity able to differentiate between clinically isolated syndrome patients with a high and a low risk of developing multiple sclerosis. Based on the combination of abundances of proteins chitinase 3-like 1 and ala-β-his-dipeptidase in cerebrospinal fluid, we built a statistical model able to assign to each patient a precise probability of conversion to clinically defined multiple sclerosis. Our results are of special relevance for patients affected by multiple sclerosis as early treatment can prevent brain damage and slow down the disease progression. *Molecular & Cellular Proteomics* 15: 10.1074/mcp.M115.053256, 318–328, 2016.
when quantifying simultaneously a panel of proteins across many different biological samples (7–9). In particular, selected reaction monitoring (SRM) is the gold standard targeted mass spectrometry method for protein quantification due to its high precision, reliability, and throughput (10–13). This targeted mass spectrometry method is performed on triple quadrupole instruments, in which a predefined peptide precursor ion is first isolated, and then selected fragment ions arising from its collisional dissociation are measured over time. Each pair of precursor and fragment ion is called a transition, and multiple transitions can be coordinately measured and used to conclusively identify and quantify a peptide in a clinical complex sample.

In a previous study, we used a screening mass spectrometric approach to discover potential markers for multiple sclerosis conversion in patients that initially presented a clinical isolated syndrome (14). In that discovery phase, quantitative mass spectrometry with iTRAQ labeling was used to measure protein abundances in pooled CSF samples from patients presenting a clinical isolated syndrome that either remained normal (CIS) or had eventually converted to clinically definite multiple sclerosis (CDMS) (n = 60). In the initial screening, several proteins exhibited significant differences in abundance when comparing these two groups of patients. The abundance change in one of the altered proteins, chitinase 3-like 1 (CH3L1), was confirmed by ELISA in CSF of individual patients, whereas for others, such as semaphorin 7A (SEM7A) and ala-β-his-dipeptidase (CNDP1), their abundance changes were confirmed by targeted mass spectrometry in follow-up studies with independent cohorts (15). Moreover, the levels of CH3L1 were associated with brain MRI abnormalities and disability progression during the follow-up period, as well as with shorter time to conversion to clinically definite multiple sclerosis (14).

We now set out to establish a diagnostic protein classifier with high sensitivity and specificity able to differentiate between patients with a clinically isolated syndrome that have either a high or a low risk of developing clinically definite multiple sclerosis over time. For this purpose, CSF samples from an independent patient cohort from the one used in the discovery study were collected, and a set of preselected protein biomarker candidates were systematically quantified by targeted mass spectrometry (SRM) and evaluated for their classification power. Out of this study, we established a protein classifier based on the combination of abundances of proteins chitinase 3-like 1 and ala-β-his-dipeptidase, which is able to differentiate with high sensitivity and specificity between patients with a clinically isolated syndrome that have either a high or low risk of developing clinically definite multiple sclerosis. Moreover, the statistical model built around this protein classifier enables clinicians to easily assign to each patient a precise probability of conversion to clinically definite multiple sclerosis (Fig. 1).

**EXPERIMENTAL PROCEDURES**

**Patients**—A patient cohort consisting of 50 patients with clinical isolated syndrome and 23 individuals with other neurological disorders was used in the present study (Table I and Supplemental Table ST1). This cohort was independent from that used in our previous discovery study (14), and samples were recruited at the Hospital Ramón y Cajal (Madrid, Spain). Patients with clinically isolated syndrome were classified according to the following criteria: no conversion to CDMS during the follow-up period, negative IgG oligoclonal bands, and 0 Barkhof criteria at a baseline brain MRI (n = 25) or conversion to CDMS, presence of IgG oligoclonal bands, and an abnormal brain MRI at baseline 2, 3, or 4 Barkhof criteria (n = 25) (15). A full description of clinical information and cerebrospinal fluid characteristics of all patients included in the present study is provided in Supplemental Table ST1. All CSF samples were collected in polystyrene sterile tubes, centrifuged at 1600 rpm for 15 min at room temperature.
Protein-based Classifier for Multiple Sclerosis

Sample Preparation and Mass Spectrometry—For each patient, 150 µl of CSF were used for protein precipitation in cold acetonitrile overnight at 4 °C. Pellets were then solubilized in 6 m urea in ammonium bicarbonate 200 mM, reduced with 100 mM dithiothreitol, alkylated with 200 mM iodoacetamide, and digested with endopeptidase LysC (2 m urea in 200 mM ammonium bicarbonate, 37 °C 16 h) and trypsin (1 m urea in 200 mM ammonium bicarbonate, 37 °C 16 h). After digestion, samples were acidified with 10% formic acid and desalted in C18 columns (micspin columns, The Nest Group Inc.). Five isotopically labeled reference peptides at C-terminal lysine ([13C6,15N2-Lys]) or arginine ([13C6,15N4-Arg]) were spiked into the desalted samples, three corresponding to SEM7A (IFAVWK; VYLFDF-), and two to CNDP1 (ALEQDLVPNK; HLEDVFSK). These reference peptides were used as internal standards for normalization and quantification purposes. Peptides were separated chromatographically (nanoHPLC, Eksigien) prior to online mass spectrometric analysis in a Q-Trap mass spectrometer (5500 Q-Trap, AB Sciex). Briefly, peptides were initially trapped in a precolumn (C18, 15 µm, 100 Å, Acclaim PepMap 100, Thermo Scientific) and then separated by reverse-phase chromatography using a 15-cm C18 column (75 µm, 300 Å, 3 µm, Nikkyo Technos) with a gradient from 2 to 40% of solvent B in 35 min at a flow rate of 300 nL/min. Solvent A: H2O, 0.1% formic acid; Solvent B: acetonitrile (ACN), 0.1% formic acid.

SRM acquisition was performed using an unscheduled targeted acquisition method with a dwell time range of 10–20 ms and a total cycle time of 1.4 s. For each peptide, at least four transitions were monitored (Supplemental Table ST2). SRM data were processed using the Skyline software v1.4.0 (44) and data peaks were evaluated based on retention time, transition intensity rank as compared with MS2 spectral library, and for proteins SEM7A and CNDP1, coelution between the endogenous and the labeled reference peptide were also considered (Fig. 2 and Supplemental Fig. S2). Acquired SRM raw data are publicly available at the PASSSEL repository with the accession number PASS00715.

Statistical Methods—SRM peak areas were normalized based on the labeled internal peptide standards using the SparseQuant MSstats module (29). Briefly, the normalization relied on internal stable isotopic labeled reference peptides for two targeted endogenous proteins, which were used to (i) equalize the median reference abundance for the two proteins across all runs, (ii) shift all endogenous areas in a run by a same bias, and (iii) impute all missing reference areas. Comparisons of relative protein abundance between groups were performed with expanded scope of conclusion for technical replication and with restricted scope of conclusion for biological replication as implemented by software package MSstats (43).

For predictive analysis, the whole patient cohort was divided into training and validation sets with 3:1 ratio. The software package MSstats (43) was then used to perform the model-based estimation of the quantity of each protein based on a relative log2-transformed. Protein quantity estimation was calculated independently for the training set and validation set. Missing quantification values were imputed with a minimum estimated log2-transformed abundance for a given protein across runs, representing the limit of detection in the training and validation set separately. Calculated relative abundances were used as input variables to a logistic regression model between groups. Within the training set, fourfold cross-validation was performed to find the most discriminative combination of proteins. Patients were divided into four subgroups with equivalent proportions in the training set. For each group, each protein was fitted in the logistic regression model between two groups and its classification ability was evaluated by area under the curve (AUC). The most discriminative protein was selected as the first classifier. Most discriminative proteins were repeatedly added while increasing AUC values. The proteins selected at least twice within the four cross-validation steps in a training set were chosen as the characteristic classification signature for that training set. The best classification signature for each training set was fitted in a logistic regression model and was applied on the validation set. The procedure from division into training and validation set to fitting the logistic model with best classification signature was repeated 500 times to assess the reproducibility of classification ability. A final consensus model was comprised of the combination of proteins, which were selected most in 500 repeats. To obtain the upper level for the predictive accuracy of the selected consensus proteins, the final model was fitted to the full dataset and the predictive accuracy was quantified using the area under the ROC curve, sensitivity, specificity, and accuracy. The estimate of variability associated with the ROC curve was obtained by plotting the 25th and the 75th quartile of the sensitivities for each value of 1-specificity obtained in the validation set over all the iterations for which the particular protein combination was selected. The pROC in R were used to draw ROCs, to calculate AUCs, and other performance (i.e. sensitivity, specificity, and accuracy).

RESULTS

Selection of Protein Biomarker Candidates—The set of proteins selected for our validation study was based on our former studies (14–16) as well as on previous reports involving certain proteins in multiple sclerosis (Table I) (16–28). Within this group of studied proteins, protein chitinase 3-like 1 (CH3L1) was included since it was the only protein for which we had previous evidence of its association with the risk of conversion to clinically definite multiple sclerosis (14). The inclusion of this protein served not only as a positive control for the differential protein abundances observed, but, more importantly, it was also an excellent candidate for an eventual biomarker protein combination.

Whenever possible and clinically relevant, protein isoforms and natural variants described for the 24 selected proteins were also included in the study, thus making a total of 32 proteoforms. The inclusion of the selected proteoforms in the final SRM assays depended on the detectability of specific peptides by SRM that could unequivocally identify them.

Quantification of the Protein Biomarker Candidates by SRM—SRM assays were designed for the 24 selected proteins and their isoforms and natural variants based on in-house spectral libraries built from tandem mass spectra. SRM assays corresponding to 1–3 unique peptides per protein (including protein isoforms and variants) were developed (Supplemental Table ST2) and used to consistently identify and quantify the targeted proteins.

CSF from patients with clinically isolated syndrome was collected at the moment of their first relapse. Patients were then enrolled in a follow-up study and eventually divided into two groups depending on their clinical evolution: (i) patients that did not develop multiple sclerosis (hereafter referred as CIS, n = 25) and (ii) patients that developed multiple sclerosis (referred to as CDMS, n = 25). Cerebrospinal fluid
samples from individuals with other neurological disorders were also included (OND, \( n = 23 \)) (Table II, Supplemental Table ST1). All individual samples were digested and analyzed with targeted nLC-SRM for protein quantification analysis. Five isotopically labeled peptides corresponding to proteins SEM7A and CNDP1 were spiked-in in each sample and later used as internal standards for intensity normalization using a sparse quantitation strategy (29). A total of 28 peptides representing 19 of the preselected proteins (23 proteoforms when including isoforms and variants) were consistently detected and quantified across all measured patients (CIS, CDMS, and OND) (Supplemental Table ST3). Two samples (patient id 47 (CDMS) and 68 (OND)) were discarded for further analyses due to chromatographic technical issues.

The identification of each peptide was based on the intensity order of transitions between the SRM peaks and the reference spectral library, the relative retention times across runs, and, in the case of SEM7A and CNDP1, on the coelution of endogenous peptide and spiked-in reference references (Fig. 2 and Supplemental Figs. S2 and S3). Protein relative quantitation was performed among the CIS, CDMS, and OND patient groups using SRM and the sparse quantitation strategy (29), in which the internal standards were used to estimate the sample variability and normalize protein levels across all SRM runs.

### Table I

| Protein     | Protein Name                     | Peptide Sequence                  | UniProt Accession |
|-------------|----------------------------------|-----------------------------------|-------------------|
| SEM7A_HUMAN | Semaphorin-7A                    | IFAWWK, VYLFDFPEGK, LQDVFPLDPGWR | O75326            |
|             |                                  |                                  | O75326            |
| HPT_HUMAN   | Haptoglobin                      | VTSIODWQK                        | P00738            |
| AACT_HUMAN  | Alpha-1-antichymotrypsin         | FNLTESEAIHQFQHQLLR, LYGSEAFTDFQDSAAK, HLLPQQSK, ITLLSALVETR | P01011-1, P01011-2, P01011-1, P01011-3, P01011-1 |
| A2MG_HUMAN  | Alpha-2-macroglobulin            | DMYSFLEDMLKL, LVAYTGLASGQR       | P01023            |
| CO3_HUMAN   | Complement C3                    |                                 | P01024            |
| CYTC_HUMAN  | Cystatin-C                       | ALDFAVQYNK                       | P01034            |
| A1AG1_HUMAN | Alpha-1-acid glycoprotein 1      | TEDTIFLR, SDVYTDWK, SDVMYTDWK     | P02763            |
|             |                                  |                                  | P02763 (natural variant) |
| TTHY_HUMAN  | Transferrin                      | GSPAINVAVVFR                     | P02766            |
| THY1_HUMAN  | Thy-1 membrane glycoprotein      | HENTSSSPIYFSLTR                  | P04216            |
| CFAH_HUMAN  | Complement factor H              | SPDVINGSPISQK                    | P08603            |
|             |                                  |                                  | P08603 (natural variant) |
| OSTP_HUMAN  | Osteopontin                      | ANDESHESDVIDSQELS                 | P10451            |
| CMGA_HUMAN  | Chromogranin-A                   | ELODLAQGAK                       | P10645            |
| CLUS_HUMAN  | Clusterin                        | ASSIIDEFQDFR                     | P10909            |
| SCG2_HUMAN  | Secretogranin-2                  | ALEYIENLR                        | P13521            |
| IL7RA_HUMAN | Interleukin-7 receptor subunit alpha | LWNIFVR                          | P16871-3          |
|             |                                  |                                  | P16871 (natural variant) |
| PON1_HUMAN  | Serum paraoxonase/arylesterase 1 | SFNPNSPGK, ILLMINDOEPTVLELGITGSK, ILLMINDOEPTVLELGVTGSK | P27169, P27169, P27169 |
|             |                                  |                                  | P27169 (natural variant) |
| CH3L1_HUMAN | Chitinase-3-like protein 1        | THGFDGLDLAWLPGR                  | P36222            |
| MUC18_HUMAN | Cell surface glycoprotein MUC18  | GATLALTQVTPQDER                  | P43121            |
| TNF10_HUMAN | Tumor necrosis factor ligand superfamily member 10 | SGIAFLCK | P50591 |
| CNTN1_HUMAN | Contactin-1                      | GTEWLWNSSR, DGEYVEVR              | Q12860, Q12860 |
| KLK6_HUMAN  | Kallikrein-6                     | LSELIQPLPLER, SSWGTSFGK          | Q92876-3, Q92876-3 |
|             |                                  |                                  | Q92876-1 (natural variant) |
| PGCB_HUMAN  | Brevican core protein            | ALHPEEPDEGR                      | Q96GW7            |
| CNDP1_HUMAN | Ala-β-his-dipeptidase            | ALEDQLPNVIK, HLEDVFSK            | Q96KN2, Q96KN2    |
| Q53X90_HUMAN| C-X-C motif chemokine            | SSSTLVVPVFK                      | Q53X90            |
The aim of the present study was to identify protein combinations able to classify patients with a clinical isolated syndrome at the moment of their first attack into those that will eventually develop clinically defined multiple sclerosis and those that will not convert. Toward this goal, all quantified proteins were challenged to correctly classify our patients with a clinical isolated syndrome into either CIS or CDMS—both individually and in combination—regardless of their exhibited abundance changes. We performed a predictor selection combined with cross-validation to select a combination of proteins with predictive ability and evaluated their performance using receiver operating characteristic (ROC) curves on a separate set of subjects (Fig. 3). More specifically, the whole cohort was randomly divided into two groups: three-fourths of the patients were used to train the classification model (training set), and one-fourth of the patients were used for validating the protein classifier sensitivity and specificity (validation set). Fourfold cross validation was performed with the training set. Within each fold, the protein classification power of each protein was evaluated by a logistic regression model first. Additional proteins were then added into the best protein classifier in a stepwise manner, and new proteins were added only if they increased the classification power of the protein classifier. Protein combination selected at least twice during the cross-validation process were set as candidate protein combinations. The training set was then used to fit the logistic regression model for candidate protein combination whereas the validation set was used to evaluate its discriminatory performance between CIS and CDMS patients. Finally, to assess the robustness of the selected candidate protein combinations, the whole cross-validation process was repeated 500 times (Supplemental Table ST5).

Protein Abundance Changes Associated with CDMS and CIS Patients—Our study was complemented with a significance analysis of the 19 proteins quantified (23 proteoforms) across the same patients by quantitative targeted proteomics to pinpoint proteins that might be involved in the development of the disease. This analysis revealed five proteins that were significantly lower in abundance in CDMS patients than CIS patients—namely CNDP1, A1AG1, KLK6, CLUS, and SEM7A—while proteins CH3L1 and AACT exhibited significantly higher protein levels in CDMS patients than CIS patients (Supplemental Fig. S4 and Supplemental Table ST4). Similarly, protein levels in both CDMS and CIS patients were also compared with patients with other neurological disorders (OND), with several proteins showing significant changes in abundance (Supplemental Fig. S5).

### Table II

| Characteristics       | CIS   | CDMS | OND  |
|-----------------------|-------|------|------|
| n                     | 25    | 25   | 23   |
| Age (years)*          | 38 (12.4) | 33 (9.3) | 45 (12.3) |
| Female/male (% female) | 16/9 (64%) | 17/8 (68%) | 14/11 (61%) |
| Years follow up*      | 3.25 (1.32) | 4.08 (2.48) | –               |
| Clinical presentation  | –     | –    | –    |
| Optic neuritis        | 8 (32%) | 3 (12%) | –    |
| Brain stem            | 3 (12%) | 7 (28%) | –    |
| Spinal                | 7 (28%) | 10 (40%) | –    |
| Others                | 7 (28%) | 4 (16%)* | –    |
| Total protein (mg/dl)*| 28.0 (11.3) | 31.0 (12.6) | 35.2 (12.9) |

* Data are expressed as Median (Standard Deviation).
Fig. 2. (A and B) Targeted mass spectrometric signals (SRM transitions) for the endogenous peptide and its corresponding reference fragmentation spectrum measured for protein CH3L1; (C–F) targeted mass spectrometric signals (SRM transitions) for the endogenous peptide and the isotopically labeled reference peptides measured for protein CNDP1; (F) retention time drift of peptides THGFGLDLAWLYPGR and ALEQDLPVNIK measured in patient samples.
Fig. 3. (A) Frequency plot representing the times that a protein combination was selected as the best classifier between CIS and CDMS patients by our statistical analysis procedure over 500 iterations. (B–D) Classification analysis of the three best protein classifiers between CIS and CDMS patients by a receiver operating characteristic analysis (CH3L1+CNDP1; CH3L1+CLUS; A1AG1+AACT-2). Black solid line is the ROC curve for logistic model with three best protein combinations between CIS and CDMS patients in whole patient cohort. Gray area is the area between 25 and 75 percentile sensitivity for certain specificity among 500 iterated validation performance, which were calculated with the model with selected protein classifier in each iteration.

Fig. 4. Patient class discrimination based on pairs of estimated log2 transformed protein abundances prior statistical modeling for CH3L1+CNDP1 (A), CH3L1+CLUS (B), and A1AG1+AACT-2 (C).
CH3L1 was found to be significantly increased in patients that became CDMS as we had demonstrated in previous studies based on immunochemistry assays (14). Using an antibody-based technique and an independent cohort from the one used in this study, higher protein levels of CH3L1 have been associated to patient conversion into clinical definite multiple sclerosis. In the present study, other protein abundance changes that were proposed in our previous screening study, such as those for SEM7A, CNDP1, and AACT—proteins for which no antibodies are available—have also been validated using targeted mass spectrometry. Moreover, targeted proteomics allowed us to explore several protein isoforms and natural variants described for the selected proteins. More specifically, we quantified two different isoforms for protein AACT, and both isoforms also showed increased levels in CDMS patients when compared with CIS patients. In contrast, CSF levels of CNDP1, SEM7A, A1AG1, KLK6, and CLUS determined by SRM were significantly decreased in CDMS patients as compared with the CIS patient group (Supplemental Fig. S5). Moreover, levels of A1AG1, KLK6, and CLUS were also significantly lower in the CIS group as compared with the OND patients group, whereas SEM7A and CNDP1 levels only showed significant differences between the two CIS types of patients, thus suggesting that the latter proteins are specifically related to the onset of multiple sclerosis.

An important number of proteins, including TTHY, CYTC, CO3, A1AG1, OST, CNTN1, KLK6, PGCB, and CMGA, showed significant differences in abundance in the CSF of patients with clinical isolated syndrome (regardless of their outcome) as compared with controls (Supplemental Fig. S5). Thus, these proteins could differentiate patients with clinically isolated syndrome from patients with other neurological diseases. Although these proteins could be just inflammatory markers, our data confirm observations from previous reports in which several of these proteins were described to be involved in multiple sclerosis (19, 20, 25, 26, 28, 30–32). Out of these proteins, only KLK6 and A1AG1 showed significant differences between CIS and CDMS patients.

**DISCUSSION**

In the present study, we used a relatively large patient cohort and quantitative targeted proteomics by SRM to es-
establish a diagnostic molecular classifier with high classification power able to differentiate between patients with a clinically isolated syndrome that have either a high or a low risk of developing multiple sclerosis over time.

In order to define a sensitive and specific diagnostic molecular classifier, quantified proteins were tested for their classification power both individually and in combination among them. We found a synergistic effect among some of the assessed proteins, resulting in an improved multiple sclerosis outcome predictive power. In particular, the CH3L1+CDNP1 combination resulted in the best protein signature in terms of classifying patients with an initial clinical isolated syndrome into high and low risk patients according to their probability to develop clinically definite multiple sclerosis.

CH3L1 is known to play a role in chronic inflammation and tissue injury (33). We as well as other groups have proposed the potential use of CH3L1 as prognostic for multiple sclerosis, based on the elevated levels of this protein in CSF samples of patients based on various proteomics screening studies (14, 34–36). More recently, CH3L1 has been proposed as a biomarker of therapeutic response because CSF CH3L1 levels were significantly reduced after 1 year of natalizumab treatment (35) and because the detected association of elevated CSF CH3L1 levels and astrogliosis (37). In our recent study, we could confirm in a large cohort the association of elevated CSF CH3L1 levels with a risk of conversion to clinically definite multiple sclerosis (14). Nonetheless, our results demonstrate that the classification power of CH3L1 is improved when its CSF levels are measured in combination with those of protein CNDP1. Carnosinase (CNDP1) hydrolyzes carnosine, which has been described to have neuroprotective effects due to its capacity to decrease oxidative stress and inflammation (38, 39). Although carnosinase activity has been associated with several neurological disorders and its potential use as a CSF biomarker has been proposed (40), its specific role in multiple sclerosis remains unknown.

Other protein combinations exhibiting high sensitivity and specificity when classifying patients with clinically isolated syndrome were CH3L1+CLUS and A1AG1+AACT-2. Clusterin (CLUS) regulates complement factors, and it is involved in oxidative stress, preventing stress-induced aggregation of secreted proteins, and beyond its CIS/CDMS classification power, this protein might also be involved in multiple sclerosis as supported by results of a recent study (41). Here, we have demonstrated that this protein improves the CIS/CDMS classification power of CH3L1. Finally, protein combination A1AG1+AACT-2 also resulted in an acceptable protein classifier but with less sensitivity and specificity than the best two protein combinations. Nonetheless, it is worth noting that the present study has confirmed that both proteins exhibit higher abundances in CDMS patients, as was previously suggested (20, 24, 42).

Based on our results, we propose a diagnostic test based on the abundance of proteins CH3L1 and CNDP1 in CSF to differentiate between clinically isolated syndrome patients with a high and a low risk of developing multiple sclerosis. More specifically, we have used the abundance of proteins CH3L1 and CNDP1 measured in this study to build a statistical model and generate probability maps to assist clinicians into the prognosis evaluation of each patient.

CONCLUSIONS

By using a relatively large patient cohort and quantitative targeted proteomics by selected reaction monitoring (SRM), our study enabled us to establish a diagnostic molecular classifier with high sensitivity and specificity able to differentiate between patients with a clinically isolated syndrome that have a high and a low risk of developing multiple sclerosis over time. Our results are relevant for early treatment of patients affected by multiple sclerosis as currently there is no clinical test that can conclusively establish the prognosis of a patient with a clinically isolated syndrome. Moreover, this study confirms the relevance of target mass spectrometry as an efficient technique for biomarker validation and for the establishment of new molecular classifiers with high sensitivity and specificity. Indeed, the capacity of targeted proteomics to quantify multiple proteins in large patient cohorts, together with a solid statistical approach, enables the assessment of a myriad of protein combinations that might exhibit a synergistic effect and, thus, the selection of a particular protein combination with a highly improved predictive power over single analyte evaluation.

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Protein-based Classifier for Multiple Sclerosis

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