P014

Clinical Validation of a Multivariate Proteomic Serum Based Assay for Disease Activity Assessments in Multiple Sclerosis

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Disclosures

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Background and Objective

- The current standard of care to evaluate disease activity (DA) and disease progression (DP) in MS patients relies primarily on qualitative radiographic and subjective clinical assessments.
  - **Validated tools to quantitatively measure and predict the level of disease activity will help address a significant unmet medical need.**

- **A custom immunoassay panel that measures the serum concentrations of up to 21 proteins** associated with biological pathways involved in MS pathophysiology **has been developed** and was analytically validated.

- Proof of concept was demonstrated by **associating proteomic profiles with clinical and radiographic endpoints in several independent discovery studies**. Biological pathway modeling and protein network analysis were performed to ensure comprehensive representation of MS neurophysiology.

- **Objective:** To present the design and analysis plan for a study that is being performed to **validate associations between this blood-based multiplex proteomic DA test with the presence and count of gadolinium enhancing (Gd+) lesions** as the primary endpoint.
  - **Samples** for this clinical validation study have been **collected from 3 sites**: **Brigham and Women's Hospital** (CLIMB Study), **University of Massachusetts** (FSDD Study), and the **American University of Beirut**.

Biomarkers Measured in the Custom Assay Panel

ANALYTE	PROTEIN NAME AND ALIAS
APLP1	Amyloid Beta Precursor Like Protein 1
CCL20	MIP-3 alpha
CD6	Cluster of Differentiation 6
CDCP1	CUB domain-containing protein 1
CNTN2	Contactin 2
COL4A1	Collagen alpha-1(IV) chain
CXCL13	C-X-C Motif Chemokine Ligand 13, BLC
CXCL9	MIG, Monokine Induced by Gamma Interferon
FLRT2	Leucine-rich repeat transmembrane protein
GFAP	Glial Fibrillary Acidic Protein
GH	Growth Hormone, Somatotropin
IL-12B	Interleukin 12B
MOG	Myelin-Oligodendrocyte Glycoprotein
NEFL	NFL, Neurofilament Light
OPG	Osteoprotegerin, TNFRSF11B
OPN	Osteopontin
PRTG	Protogenin
SERPINA9	Serpin Family A Member 9
TNFRSF10A	TRAILR1, DR5 - Death Receptor 5
TNFSF13B	BAFF, B-cell activating factor
VCAN	Versican, Versican Proteoglycan

Assay Methodology

- The **blood-based multiplex proteomic DA test is a custom designed immunoassay** panel that utilizes Proximity Extension Assay (PEA) technology and is **performed using the Olink™ platform** for bioanalytics.
- The Olink™ platform enabled access to an extensive protein library with minimal sample requirements for earlier R&D studies and the ability to develop a 21-plex focused panel that **met stringent analytical performance specifications**.
- A **fit-for-purpose analytical validation study has been performed on the custom panel** to characterize the following parameters for the individual proteins: Accuracy, Precision (inter- and intra-assay), Robustness (instrumentation and analysis location), Sensitivity (limits of detection and quantification), MS population reference ranges, Interference (endogenous interfering substances, heterophilic antibodies, common drugs and DMTs), Diurnal variability, Cross-reactivity and Stability of reagents and serum samples. (**See Poster P010 ACTRIMS 2021 for further details and results**)
  - The **analytical validation study was performed across 2 manufactured lots** (e.g batches of assay kits) for which the critical reagents (paired antibodies used for analyte capture/detection and protein stocks for calibration) were varied to the extent possible.
  - The **clinical validation study will be performed using the 2nd lot** of manufactured reagents.

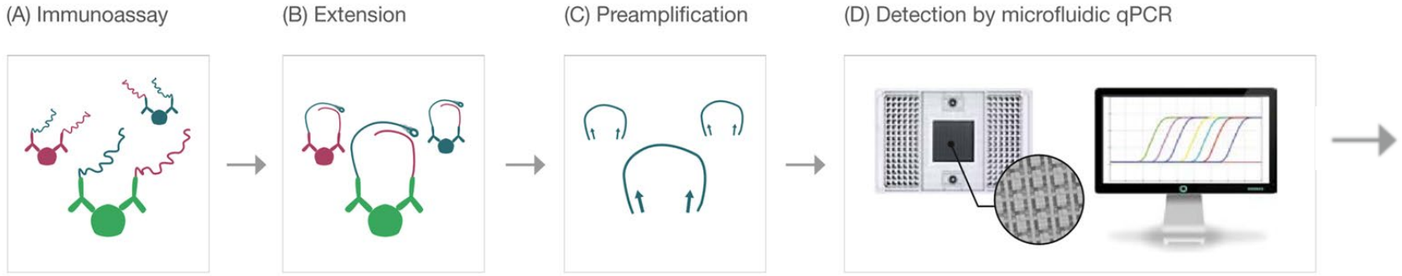
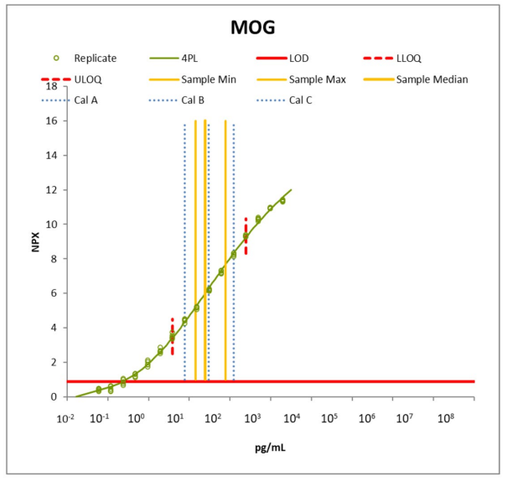


Fig 1. Overview of the PEA technology. (A) 92 Antibody pairs, labelled with DNA oligonucleotides, bind target antigen in solution. (B) Oligonucleotides that are brought into proximity hybridize, and are extended by a DNA polymerase. (C) This newly created piece of DNA barcode is amplified by PCR. (D) The amount of each DNA barcode is quantified by microfluidic qPCR.



A multiplexed standard curve was prepared along with 3 levels of Calibrators (High Mid, Low) to cover the range of sample response in the MS population. The 4PL standard curve is re-established for each lot of reagent kits during manufacturing. The calibrators and a blank are run on each assay plate. The signal obtained from the assay (NPX) is converted to absolute concentration (pg/mL) using the calibrators referenced back to the "gold" standard curve. These concentrations are then used as inputs into algorithms corresponding to Disease Activity and MS Disease Pathway Scores.

Disease Activity Score and Disease Pathways Scores

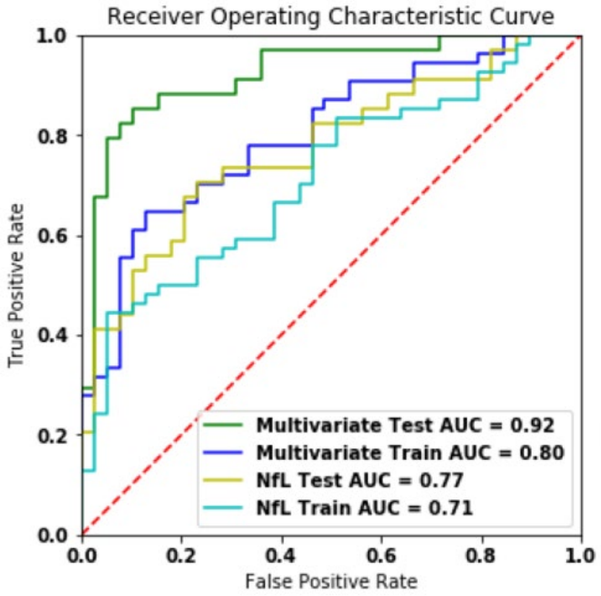
- An overall Disease Activity Score algorithm and 4 Disease Pathway algorithms will be optimized with finalized coefficients in this Clinical Validation Study.
- The Disease Activity Score algorithm will use the best performing subset of the 21 biomarkers that associate with Gd+ lesion presence and count.
- The 4 Disease Pathway algorithms are restricted to biomarkers that have been associated with the following 4 key hallmarks of MS pathophysiology according the Venn Diagram below.

0 Collect Raw Absolute Concentrations (pg/mL)

The custom assay reports out absolute quantitation for up to 21 proteins.

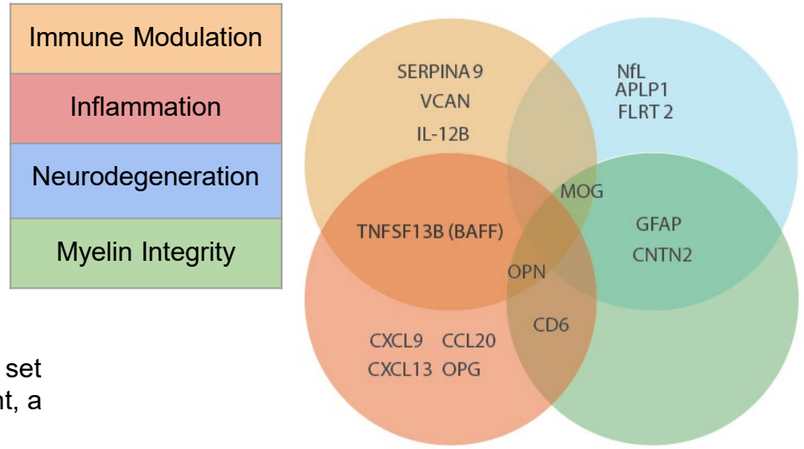
1 Log base-10 Transformation

To normalize the dataset, the base-10 logarithm is taken for each value.



2 Demographic Adjustment

Results are demographically adjusted based on age and sex, referencing a set of Non-Gad+ MS samples. Once multiple samples are available for a patient, a baseline is established to examine changes relative to earlier blood draws.



3 Weighted Combination

$$\sum_i c_i * log[protein_i]$$

The coefficients of the Disease Activity and Disease Pathway Algorithms finalized in the Train/Cross Validation Subset are used to combine information across biomarkers into a statistical model (preliminary example shown).

Preliminary Independent Holdout Model Performance Evaluation

- Train on CLIMB Cohort (n=186)
- Test on Basel Cohort (n=146)

(See Kuhle et al., P0055-MS Virtual 2020)

In earlier R&D studies, a **multivariate model** using biomarker concentrations derived from the custom assay panel trained and then **tested in independent cohorts demonstrated significant improvement** compared to the top performing univariate biomarker (**AUC 0.92 vs 0.77**) to classify patient longitudinal sample pairs with increasing (Gd- → Gd+) vs. decreasing (Gd+ → Gd-) Lesion Burden.

4 Conversion to a 10 point scale

$$P = \frac{e^{a+bX}}{1+e^{a+bX}}$$

The equation on the right is then used to transform the multivariate classifier into a confidence score reflecting the likelihood that a sample has Gd disease activity. In the first implementation, we multiply by 10 then round and rescale to the nearest half point between 0-10 (analogous to EDSS scaling).

Sample Characteristics

Table 1: Estimated Sample Count from Each Participating Site	
Brigham and Women's Hospital (BWH)	200
American University of Beirut (AUB)	200
University of Massachusetts (UMMS)	26

Table 2: Targeted Distribution of Gadolinium Enhancing Lesions on a MRI administered within +/- 20 days of the serum draw				
Gd+ Lesion Count	**0 lesions**	**1 lesion**	**2 lesions**	**≥3 lesions**
Sample (N)	172	129	86	43

- Serum samples from **three deeply-phenotyped cohorts associated with Summit MS** - International Multiple Sclerosis Research Consortium **are being sourced for this study**.
  - Samples from an ongoing prospective MS Disease Activity study may also be included to increase the total sample N.
- Samples will **target a distribution reflecting the annotated count of gadolinium enhanced lesions (Gd+) on the associated MRI** according to Table 2.
  - Balanced distribution across the 3 sites for Gd+ count
  - This categorization will be used to indicate the level of Disease Activity for the patient at the time of the serum draw.
- Both **cross-sectional and longitudinal samples from patients will be included**. For longitudinal samples, a Gd+ count of 0 is required for at least one of the timepoints.
  - Longitudinal samples are to be within 18 months of at least one other sample in the series.
  - Sequential samples with 0 lesions will have no radiographic or clinical indication of a relapse between the serum draws.
  - Stable therapy between timepoints.
- Selection to **reflect demographic characteristics for MS patients regarding age, gender and ethnic background**.
  - Balanced DMT usage across entire cohort.
- **Exclusion criteria**:
  - Current (or previous 90 day) steroid use
  - Patients with current infection
  - Recent vaccination (less than 30 days)
  - Major comorbidities (including diabetes, other autoimmune diseases, other inflammatory diseases, cancer and neoplasia).

Clinical and Radiographic Data

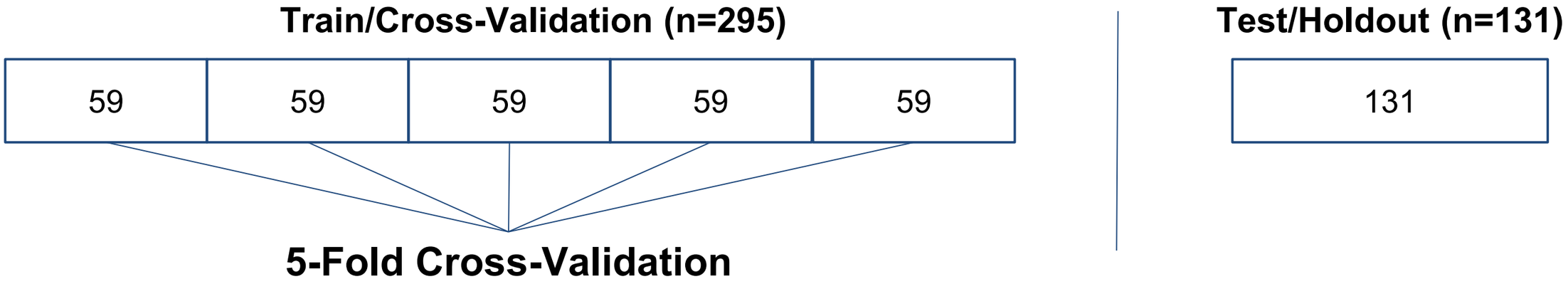
The following **de-identified clinical and radiographic data** fields will be collated across the three cohorts for the **biostatistical analysis**:

- Sample/Subject ID
- Age (year with decimal)
- Sex – M/F/other
- Race and Ethnicity – NIH categories
- Height, Weight, BMI within 1 year of sample
- Smoking Status – ever/never
- Date of First Symptom – age (year with decimal)
- Date of MS Diagnosis – age (year with decimal)
- MS Subtype – CIS, RRMS, SPMS, PPMS
- Relapse features:
  - Relapse dates – age (year with decimal)
  - Symptoms – optic neuritis, transverse myelitis, brainstem symptoms, cerebral symptoms, etc.
- EDSS within 6 months of sample
- Age at Current DMT age (year with decimal)
- Age at Previous DMT age (year with decimal)
- Last Steroid Use (IV or oral steroid)
- Serum sample age (year with decimal)
- MRI age (year with decimal)
- # of days between sample draw and MRI
- Brain Gd+ lesion count (and volume when available)
- Additional radiographic features:
  - Spine Gd+ lesion count (if applicable)
  - T2 lesions
  - New and Enlarging T2 lesion count (and volume if available)
  - BPF (if available).

*The **Primary Endpoint for this Clinical Validation Study is the association of proteomic profiles with the presence and count of Gd+ enhancing lesions**. Additional Clinical and Radiographic data pertaining to Disease Activity and Disease Progression assessments will be utilized for exploratory endpoint analyses.*

Randomization and Stratification

- The total **sample count** available upon initiation of the analytical phase **will be split into two subsets**:
  - (1) **Train/Cross-Validation Subset** to optimize algorithms = **70% of total available samples**
    - This subset will be further partitioned to allow for a balanced **K-fold Cross-Validation**
  - (2) **Test/Holdout Subset** = **30% of total available samples**
- Each subset will be **randomly stratified across the sites, demographics, and for the primary endpoint (Gd+ lesion count)**.
  - Balanced representation **subject to final cohort characteristics** upon sample acquisition.
  - This approach will be used to refine final Disease Activity and Disease Pathway Algorithm(s)
- **Analytical runs will be stratified as well**, ensuring assay plates have balanced distributions across the sites, demographics and the primary endpoint.
  - Longitudinal samples from the same subject will be included on the same assay plate.
  - Post-assignment, samples within each immunoassay plate will be randomized on the plate map (e.g. well location).
- Primary statistical analysis will be performed by Octave.
  - An independent statistical group will repeat the analysis for verification purposes.



Statistical Analysis Plan

- Associations with **disease activity** will be determined based on the presence (or lack thereof) and count, of **gadolinium enhancing lesions** as determined on a matched MRI (administered within +/- 20 days of the serum draw).

- **Demographic data** that is expected to be **available in a clinical setting** (including **age and sex**) will be **incorporated as covariates** in the analysis.
  - Impact of **additional clinical variables** (i.e. disease duration, DMT use, relapse history, etc.) **will be characterized.**

- Both **classification and regression models** related to radiographic disease activity will be fit to the proteomic results.
  - Area under the receiver operating characteristic curve (**AUC**), **sensitivity**, **specificity**, **accuracy**, and **F1 score** are the statistical metrics for classification model performance evaluation.
  - **Adjusted R^2** and **root mean squared error** are the key metrics for characterizing the performance of regression models.
  - **Regularization** methods will be used to minimize overfitting when training and ensure generalizability to the test set.

- **Multivariate models** will be **compared to univariate protein performance** using p-values to demonstrate significantly improved classification and regression performance.

- **Exploratory endpoints** will include association of biomarker signatures with additional clinical and radiographic endpoints related to disease activity, disease severity and disease progression including: **EDSS, New and Enlarging T2 lesions, BPF,** and **clinical relapse status**.

- Analysis will be performed **cross-sectionally** (i.e. concentrations as predictors) as well as **longitudinally** (i.e. shifts in biomarker values between timepoints as algorithmic features).

Conclusion and Next Steps

- The design of this **multi-site clinical validation study** is intended **to finalize algorithms related to Disease Activity and MS Disease Pathways** that maximize performance relative to aforementioned endpoints and **ensure generalizability to the broader MS population**.
- The results of a concurrent **analytical validation study will complement this clinical validation** and provide assurance that the **assay has been thoroughly characterized** with reported **results that are accurate, precise and robust.**
    - Upon completion of the clinical validation study, the **final Disease Activity and Disease Pathway Algorithms** that utilize ensembles of proteins for the reported output **will also be assessed for the analytical validation parameters.**
- A **validated** multivariate proteomic **blood-based assay for DA assessments will serve as a quantitative and objective tool** available to physicians to **enhance the care for MS patients**.
    - **Proposed Clinical Utility** for a Validated DA Test: (1) Identification of active relapse, (2) Prediction of impending relapse, (3) Confirmation of NEDA status, (4) Assessment of longitudinal changes relative to previous tests, (5) Response to DMTs
        - **Expansion of the tests clinical utility to be investigated with future studies** to evaluate biomarker correlations with endpoints associated with Disease Progression, Therapy Selection and Differential Diagnosis.

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