Peptides from the variable domain of immunoglobulin G as biomarkers in Chronic Inflammatory Demyelinating Polyradiculoneuropathy

Joris Godelaine, PharmD[1,2]; Yamini Chitale, M.Sc.[3]; Bart De Moor, PhD[3]; Chantal Mathieu, PhD, MD[4,5]; Lina Ancheva, PhD[6]; Philip Van Damme, PhD, MD[7,8]; Kristl G. Claeys, PhD, MD[7,9]; Xavier Bossuyt, PhD, MD[2,6]; Sebastien Carpentier, PhD[10]; Koen Poesen, PhD, PharmD[1,2]

[1] Department of Neurosciences, Laboratory for Molecular Neurobiomarker Research, Leuven Brain Institute, KU Leuven, Leuven, Belgium

[2] Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium

[3] STADIUS Center for Dynamical Systems, Signal Processing, and Data Analytics, Department of Electrical Engineering (ESAT), KU Leuven, Leuven, Belgium.

[4] Department of Endocrinology, University Hospitals Leuven, Leuven, Belgium.

[5] Department of Chronic Diseases and Metabolism, Clinical and Experimental Endocrinology, KU Leuven, Leuven, Belgium

[6] Department of Microbiology, Immunology and Transplantation, Clinical and Diagnostic Immunology, KU Leuven, Leuven, Belgium

[7] Department of Neurology, University Hospitals Leuven, Leuven, Belgium.

[8] Department of Neurosciences, Experimental Neurology, Laboratory of Neurobiology, Leuven Brain Institute, VIB KU Leuven Center for Brain and Disease Research, Leuven, Belgium

[9] Department of Neurosciences, Laboratory for Muscle Diseases and Neuropathies, and Leuven Brain Institute, KU Leuven, Leuven, Belgium

[10] Department of Biosystems, Division of Crop Biotechnics, Tropical Crop Improvement Laboratory, KU Leuven, Leuven, Belgium

Correspondence to: Prof. Dr. Koen Poesen, Laboratory for Molecular Neurobiomarker Research, KU Leuven (University of Leuven), 3000 Leuven, Belgium; 0032 16347013; koen.poesen@uzleuven.be

Abstract

Backgrounds and objectives: Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP) is a clinically heterogeneous immune-mediated disease. In the majority of patients with CIDP, biomarkers are currently lacking. Peptides derived from the variable domain of circulating immunoglobulin G (IgG) antibodies have earlier been shown to be shared among patients with the same immunological disease. As humoral immune factors are hypothesized to be involved in the pathogenesis of CIDP, we evaluated IgG variable domain-derived peptides as diagnostic biomarkers in CIDP (primary objective) and whether IgG-derived peptides could cluster objective clinical entities in CIDP (secondary objective).

Methods: IgG-derived peptides were determined in prospectively collected serum of patients with CIDP and neurological controls by means of orbitrap mass spectrometry. Peptides of interest were selected via statistical analysis in a discovery cohort followed by sequence determination and confirmation. Diagnostic performance was evaluated for individual selected peptides as well as for a multipeptide model, followed by performance reassessment in a validation cohort. Clustering of patients with CIDP based on IgG-derived peptides was evaluated through sparse principal component analysis followed by k-means clustering.

Results: 16 peptides originating from the IgG variable domain were selected as candidate biomarkers in a discovery cohort of 44 patients with CIDP and 29 neurological controls. Univariate logistic regressions and ROC-curve analysis demonstrated all selected peptides to be predictors of CIDP (AUCs ranging from 64.6% to 79.6%). 13/16 peptides remained predictors of CIDP when including age and sex as covariates in logistic regression models. A model comprised of 5/16 selected peptides showed strong discriminating performance between CIDP and controls (AUC 91.5%; 95% CI, 84.6% to 98.4%; p <0.001). In the validation cohort containing 45 patients and 43 controls, two peptides remained significant predictors of CIDP in logistic regression models while the five-peptide model demonstrated an AUC of 61.2% (95%

CI 49.3% to 73.2%, $p = 0.064$). Peptide-based patient clusters derived through unsupervised clustering did not associate with clinical characteristics.

Discussion: IgG variable domain-derived peptides showed a valid source for diagnostic biomarkers in CIDP, albeit with challenges towards replication. Our findings warrant further research of IgG-derived peptides as biomarkers in homogeneous cohorts of CIDP and controls.

Introduction

Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP) is a neuroinflammatory disorder demonstrating autoimmune responses against peripheral nerves. With a reported prevalence of 0.7 to 10.3 cases per 100 000 people it is rare disease that shows considerable variation in clinical phenotype and therapy response among patients.[1] Due to this large clinical heterogeneity, CIDP is often misdiagnosed or underdiagnosed despite diagnostic criteria being available.[2-5] While highly specific paranodal antibodies have been described in small subsets of patients initially considered to be CIDP, biomarkers applicable to the broader population of patients that can aid in diagnosing CIDP are still lacking.[6,7] Nevertheless, both the discovery of paranodal antibodies as well as earlier reports indicating antibody responses directed against Schwann cells or compact myelin in up to 40% of patients with CIDP suggest circulating antibodies a candidate source for future discovery of novel CIDP biomarkers.[8,9]

A relatively novel approach in biomarker discovery for immune-mediated diseases is mass spectrometric (MS) analysis of peptides derived from circulating immunoglobulin G (IgG). Such peptides might be specifically or more abundantly present in patients compared to controls and, hence, could possibly provide interesting diagnostic biomarkers. This MS-based approach allows an unbiased study of circulating antibodies independent of the targeted antigen. Moreover, in contrast to classical techniques such as organic substrate assays, it also allows differentiating between antibodies displaying minor structural differences that target the same antigen. This could be relevant as small differences in structure have previously been shown to potentially affect pathogenicity of an antibody.[10-13]

Human IgG antibodies are composed of two light and two heavy chains. Light chains consist of one variable and one constant domain while heavy chains have one variable and three different constant domains.[14] The variable parts together with the first constant domain form the antigen binding fragment wherein a set of three complementary determining regions (CDRs)

embedded in framework regions (FRs) form a groove that fits the epitope of an antigen.[14] As such, CDRs determine the antigen specificity of the immunoglobulin. Due to processes such as somatic recombination and hypermutation, the human antibody repertoire is extremely diverse with the total number of antibody specificities estimated at more than 10^{11}.[15] Hence, theoretically, one would expect it unlikely to find common sequences in IgG variable domains among different individuals. Nevertheless, in recent years, multiple research groups have shown overlapping variable domain-derived sequences in patients with the same neuroimmunological disorder including multiple sclerosis or paraneoplastic neurological syndromes.[16-19] Moreover, a panel of variable domain-derived peptides already demonstrated being capable of differentiating patients with lung cancer from controls, thereby demonstrating the biomarker potential of such peptides.[16]

As the humoral immune response is considered an important factor in the pathogenesis of CIDP, studying IgG-derived peptides may also provide novel insights with respect to biomarker research in CIDP.[5] Therefore, the aim of this study was to investigate the IgG-derived peptide profile in CIDP by means of Orbitrap MS to determine i) whether serum of patients with CIDP contains clusters of IgG-derived peptides capable of differentiating CIDP from controls and ii) whether individual variable domain-derived peptides in these clusters or a panel thereof demonstrate potential as diagnostic biomarker in CIDP (primary objectives). We also investigated whether IgG-derived peptide profiles could cluster patients in the CIDP population and whether these clusters differed in clinical features (secondary objective).

Methods

Study population

Adult patients with neuromuscular disorders were prospectively recruited at the University Hospitals of Leuven Neuromuscular Reference Center upon written informed consent. Serum of patients with CIDP or related neurological controls (Guillain-Barré syndrome (GBS), anti-myelin-associated glycoprotein-related neuropathy (MAG), monoclonal gammopathy of undetermined significance-related neuropathy (MGUS), multifocal motor neuropathy (MMN), Charcot-Marie-Tooth IA neuropathy (CMTIA), diabetic peripheral neuropathy (D-PNP) and amyotrophic lateral sclerosis (ALS)) was collected between April 2014 and July 2022, aliquoted and stored at -80°C until use. Sampling occurred before initiation of immunomodulatory therapy or, when a patient was already receiving therapy at time of recruitment, at trough levels (i.e. before next administration of therapy). Diagnoses were established by trained neurologists. For CIDP diagnosis, patients had to fulfill probable or definite CIDP criteria according to the 2010 EFNS/PNS diagnostic criteria.[2] Patients with CIDP were seen at our reference center for diagnostic work-up or, as required for Belgian legislature for reimbursement of intravenous immunoglobulin (IVIg) therapy, for confirmation of CIDP diagnosis or related periodic follow-up . Relevant clinical data was obtained via electronic medical records and included demographics (age, sex, disease duration, CIDP phenotype), disability at time of sampling (via the Inflammatory Neuropathy Cause and Treatment (INCAT) scale and an 80-point Medical Research Council (MRC) sum-score) and therapy received at time of sampling.

Study design

We first investigated in a discovery cohort i) whether IgG-derived peptide clusters could differentiate patients with CIDP from controls and ii) whether individual IgG variable domain-derived peptides showing promise as diagnostic CIDP biomarkers were present (further termed peptides of interest (POIs)) (primary objectives). Next, as an amino acid sequence was not obtained for all POIs selected during the discovery phase, targeted MS was performed to obtain higher quality MS/MS spectra of POIs thereby allowing elucidation of their amino acid sequences. Sequences obtained in this manner were confirmed against synthetically constructed peptides (Thermo Scientific). Next, diagnostic performance of individual POIs as well as of a multipeptide model incorporating POIs was evaluated in the discovery cohort and subsequently reassessed in an independent validation cohort (Figure 1). We also investigated whether IgG-derived peptide profiles allowed to establish objective clinical clusters of patients with CIDP within the discovery cohort and whether such clusters related to specific clinical characteristics (secondary objective).

IgG isolation and in-solution digestion

IgG was isolated from serum using Magne® Protein G magnetic beads (Promega, USA) according to manufacturer's instructions with minor modifications. Twenty microliters of beads were incubated with 500 µL diluted serum (1:250 with TBS1X) for 1h followed by washing the beads three times with 2M ammonium acetate/TBS1X and two times with TBS1X. Next, IgG was eluted from the beads over 30 minutes using 50 µL of 0.2% formic acid. The elution fraction was subsequently neutralized in vials containing 1M Tris pH 8. After adding 8M urea, disulfide bonds were reduced using 70mM DTT at 37°C for 1h following alkylation with 220mM iodoacetamide for 30 minutes in the dark. Next, digestion at 37°C was performed

overnight using Pierce™ Lys-C protease (Thermo Scientific) followed by a 4h digestion with Pierce™ Chymotrypsin protease (Thermo Scientific). All steps were performed while shaking at room temperature unless specified otherwise. Lastly, digested samples were desalted using C18 spin columns (Thermo Scientific) according to manufacturer's instructions followed by drying in a SpeedVac and storage at -80°C until LC-MS/MS analysis.

LC-MS/MS analyses

Desalted peptides were dissolved in 50µL of 0.1% formic acid/ 5% acetonitrile and five microliters injected and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) online coupled to an Orbitrap Elite Velos Pro ETD mass spectrometer (Thermo Scientific) operating in data-dependent acquisition (DDA) mode. For further settings, we refer to eMethods. Samples were randomized before measurement and each sample analyzed once. Before each sample, a blank to monitor system background and reduce carry-over was run. Every third sample, an additional 2h wash step was performed. Quality control samples (BSA control (discovery cohort 1) or designated patient sample (validation cohort)) were analyzed at regular intervals during the LC-MS/MS run. Replicates of a designated patient sample were also used to evaluate reproducibility of LC-MS/MS measurement of selected POIs (eMethods). Synthetic versions of POIs (Thermo Scientific) were spiked in QC samples of the validation cohort to ensure identification of POIs during further data-analysis.

To obtain amino acid sequence information for POIs not sequenced in the discovery phase, targeted mass spectrometry was performed on discovery cohort samples demonstrating the highest abundance for POIs using an Orbitrap Q Exactive mass spectrometer (Thermo Scientific) (eMethods).

MS data analyses

Raw data files were imported into Progenesis v4.2 label-free quantification software (Nonlinear Dynamics, New Castle, UK) for alignment, label-free quantification and normalization of the different sample runs. The software automatically selects the run with greatest similarity to all other runs as the reference alignment. Peaks (features) with charge state two to five were retained. Tandem mass spectra were extracted by Progenesis and processed via Mascot (version 2.2.06; Matrix Sciences) against an in-house database (consisting of the NCBI homo sapiens database supplemented with IgG-related sequences obtained from the international ImMunoGeneTics information system (IMGT) database) using the following search parameters: peptide mass tolerance of 12 ppm; fragment mass tolerance of 0.5 Da; a maximum of four missed cleavages; Lys-C + chymotrypsin (FYWKL |) as enzyme; oxidation of M and deamidation of N/Q as variable modifications; carbamidomethylation of cysteines as fixed modification. *De novo* sequencing was also performed on extracted mass spectra using the same search parameters utilizing PEAKS Studio 5.1 software (Bioinformatics solutions Inc., Waterloo, ON, Canada). Search results from both Mascot and PEAKS were reintroduced into Progenesis to generate a peptide database containing both peptide information (m/z, sequence,…) and peptide abundances for each sample included. Amino acid sequences of POIs were also aligned to V, D, J or C-region germline sequences derived from the IMGT database using the IgBlast algorithm.[20,21] Peptides with sufficient match (bitscore ≥12.5 and alignment score ≥70%) to the V-region database were assigned a position on the immunoglobulin molecule.[16]

Selection of peptides of interest

The peptide database of the discovery cohort was exported from Progenesis and subjected to statistical analysis. First, multivariate partial least-squares discriminant analysis (PLS-DA) using the NIPALS algorithm was performed to test our initial hypothesis that IgG-derived peptide clusters can differentiate patients with CIDP from controls (dependent variable: CIDP or control; independent variables: peptide abundances). Then, to select individual POIs, the PLS-DA analysis results were further used to retain only peptides significantly contributing to differentiating CIDP patients from controls, based on Variable Importance in Projection (VIP) scores and X-loadings. Variables with a VIP-score greater than 1 were considered to have a significant effect on the classification of sample categories.[22] Next, only peptides showing significant upregulation in CIDP by univariate analysis were further retained, applying either an unpaired t-test or Mann-Whitney U test based on normality of data. A further selection of peptides was then made based on the standardized abundances observed in patients with CIDP. In detail, for each thus far retained peptide and each sample we expressed an inter-z-score indicating how many SDs the abundance of that sample differed from the mean abundance observed in samples of controls (i.e. an inter-z-score of +1 indicates a sample to have an abundance of 1SD above the mean observed in controls). Only peptides for which in at least 10% of CIDP samples an inter-z-score of +3 or higher was observed were retained as possible POIs.[23,24] The 10% cut-off was chosen in line with previous findings regarding paranodal antibodies which are found in approximately 1-10% of patients initially diagnosed with CIDP. Inter-z-scores of selected POIs were also inspected to ensure that peptides were not specifically upregulated in only those patients receiving IVIg at time of sampling. Lastly, a final selection was performed based on peptide sequence. Only peptides for whom the amino acid sequence could be determined via targeted mass spectrometry and confirmed against synthetic peptides

(eMethods), and that were shown to originate from the IgG variable domain were retained as POIs.

Statistical analyses

For continuous variables, demographics within and between cohorts were compared via unpaired t-test or Mann-Whitney U test based on normality of data. Categorical demographics were compared via Fisher's Exact tests. Pearson or Spearman correlation was used to investigate correlations of peptide abundances with clinical data such as disability scales.

Univariate and multivariable (including demographics) logistic regressions were performed to evaluate whether a POI could differentiate patients with CIDP from controls in the discovery cohort. Univariate ROC-curve analysis was also performed to report diagnostic performance of individual peptides in terms of AUCs. POIs were also incorporated into a multipeptide model by means of stepwise multiple logistic regression utilizing a normalized dataset (details on model construction are available in eMethods). To validate POIs, their diagnostic performance was re-evaluated in an independent validation cohort using similar methodologies (univariate and multivariable logistic regression, multipeptide model). The clustering of patients with CIDP based on IgG-derived peptides in the discovery cohort and correspondence to any clinical traits (secondary objective) was investigated by means of dimensionality reduction through sparse principal component analysis (sPCA) applied to the complete peptide dataset followed by k-means clustering into three patient clusters.[25]

Statistical analyses were carried out using Statistica 14 (TIBCO, California, USA), Graphpad Prism V9.0 (GraphPad Software, California, USA) and R (version 4.2.2). *p*-values of 0.05 were considered statistically significant.

Standard Protocol Approvals, Registrations and Patient Consents

Written informed consent was obtained from all participants included in the study. Ethical approval was granted by Ethical Committee Research UZ/KU Leuven (S62265).

Data availability

Data not published within this article is available at the University Hospital of Leuven or will be shared in an anonymized manner on request from any qualified investigator, subject to local and European regulations.

Results

Clinical characteristics of the study population

44 patients with CIDP and 29 controls with related neurological disorders were included in the discovery cohort, while the validation cohort comprised of 45 patients with CIDP and 43 neurological controls (Table 1). In both cohorts, the CIDP group displayed a higher proportion of males, reflecting the known gender ratio of the disease[26], and of patients treated with immunomodulatory therapy at time of sampling (IVIg, steroids or immunosuppressive drugs). In the discovery cohort, patients with CIDP were also older compared to controls ($p = 0.029$). When comparing only the CIDP groups, patients of the validation cohort demonstrated longer disease duration as well as a shorter time since last IVIg treatment (respectively $p = 0.043$ and $p = 0.005$). Patients with CIDP displayed no paranodal antibodies against contactin-1, neurofascin-155 or neurofascin-186.

Peptide clusters in the discovery cohort

After LC-MS/MS data analysis, a total of 130310 peptide features were detected in the discovery cohort. PLS-DA analysis performed on these features could group patients with CIDP thereby separating them from controls, hinting at the presence of specific IgG-derived peptides (clusters) in serum of patients with CIDP (Figure 2). The distinction between patients with CIDP and controls related to a small portion of peptide features as extracted components explained a small percentage of the variance observed in our dataset (Figure 2).

Selected peptides of interest

Peptide features measured in the discovery cohort were subjected to PLS-DA analysis as mentioned above, followed by filtering via univariate statistical analysis and a selection based on a required inter-z-score of $\geq +3$ in at least 10 percent of patients with CIDP. Peptides selected utilizing this workflow were subsequently subjected to targeted mass spectrometry for elucidation of amino acid sequences after which sequences were confirmed against synthetically constructed peptides. In total, 16 peptides shown to derive from the IgG variable domain were retained as potential POIs. All selected peptides demonstrated positive inter-z-scores in multiple CIDP patients, with inter-z-score profiles of the four most promising peptides shown in Figure 3. Inter-z-score profiles of the other retained POIs are shown in eFigure 1. An overview of retained peptides can be seen in Table 2 with additional details in eTable 1. Abundance of selected POIs showed no correlation with age or disease duration and did not differ between untreated patients and patients who were receiving immunomodulatory therapy at time of sampling. Of interest, the abundance of peptide TISRDNAQNSLY correlated with the MRC sum-score in patients with CIDP (Spearman $\rho = -0.43$; $p = 0.003$) (eFigure 2). Replicate measurement of a designated patient sample indicated that selected POIs could be

measured with acceptable reproducibility with an average intra-run and inter-run CV of 12.4% and 15.3%, respectively (eFigure 3).

Diagnostic performance of individual selected peptides of interest

The 16 retained peptides were further evaluated in the discovery cohort through univariate and multivariable logistic regression models as well as by univariate ROC-curve analysis. In univariate models, increasing peptide abundances associated with increased odds of CIDP for all peptides, with ROC-curve analysis demonstrating AUCs ranging from 64.6% to 79.6% based on the Youden index (Table 3). When including age and sex as covariates, multivariable logistic regression models demonstrated 13/16 peptides to remain predictors of CIDP diagnosis in the discovery cohort (Table 3; full multivariable models available in eTable 2). Also, when evaluating diagnostic performance of the POIs through their inter-z-score profiles, the four most promising POIs taken together could identify 33/44 (75%) CIDP cases at a fixed specificity of 100% per peptide (i.e. a cut-off per peptide equal to the maximum inter-z-score observed in controls for that peptide). Upon combining the six most promising peptides, this rose to 79.5% (35/44) of cases while all 16 POIs taken together could identify 37/44 (84.9%) CIDP cases at a fixed specificity of 100% per peptide (eFigure 4).

Diagnostic performance of a multipeptide model

Stepwise multiple logistic regression performed on selected POIs resulted in a multipeptide model comprised of five POIs, namely peptides 3, 8, 12, 14 and 16 (Table 2) (eTable 3). When applied to the discovery cohort, this model demonstrated a Youden index-based AUC of 91.5% (95% CI, 84.6% to 98.5%; $p < 0.001$) (eFigure 5).

Validation of individual POIs and the multipeptide model

To validate POIs, statistical analyses regarding diagnostic performance were repeated with peptide abundances of the POIs measured in an independent validation cohort. In univariate logistic regression, peptide SPSFQGQVTISADK remained a predictor for CIDP, although ROC-curve analysis performed for this peptide in the validation cohort now demonstrated diminished discriminating performance (Table 4). In the multivariable models, peptide IVLTQSPATL remained a predictor for CIDP (Table 4; full multivariable models available in eTable 4). The diminished diagnostic performance for most peptides was also apparent in inter-z-score profiles of the validation cohort (eFigure 6). The multipeptide model constructed in the discovery cohort comprised of five POIs now displayed a Youden index-based AUC of 61.2% (95% CI, 49.3% to 73.2%; $p = 0.064$).

Clustering of patients with CIDP based on IgG-derived peptides

sPCA was utilized to reduce the dimensionality of the full discovery cohort peptide dataset to five components consisting of eight peptides each. K-means clustering was then performed on these components resulting in three clusters of 22, 16 and six patients with CIDP, respectively. Established clusters demonstrated no differences in age, sex, disease duration, CIDP phenotype, INCAT score, MRC sum-score, number of patients treated with immunomodulatory therapy or time since IVIg treatment (eTable 5).

Discussion

In current study, we utilized LC-MS/MS to investigate IgG-derived peptides in serum of patients with CIDP and related neurological controls in two independent cohorts. In our discovery cohort, we were able to show by PLS-DA that IgG-derived peptide clusters could differentiate patients with CIDP from neurological controls. These findings possibly indicate an at least partly shared humoral immune response among patients with CIDP. Moreover, examination of individual peptides within these clusters further yielded 16 peptides originating from the IgG variable domain that demonstrated diagnostic performance for CIDP as evidenced by univariate logistic regression models and ROC-curve analysis. Most of these peptides also retained their performance when including age and sex in the models, two confounding factors shown to influence the immune response in CIDP.[27] When incorporating five POIs into a multipeptide model via stepwise logistic regression, an AUC of 91.5% was obtained, further illustrating selected IgG variable domain-derived peptides to display strong discriminating performance between patients with CIDP and neurological controls in the discovery cohort.

Of interest, most peptides selected in our study originated from the FRs although we initially hypothesized that rather peptides derived from the hypervariable CDRs would be unraveled as candidate biomarkers. Similar observations were made in an earlier study on lung cancer, in which it was hypothesized that since FR-peptides carry fewer mutations relative to the germline compared to CDR-peptides they are more likely to be present in several antibody clones therefore displaying a higher abundance which, in turn, favors their detection by LC-MS/MS.[16] An alternative explanation is that hypermutated CDRs are not as likely to be shared between multiple patients as previously put forward.[16,18] This has been illustrated by studies that e.g. found only few CDR-derived peptides to be shared in only a small number of patients with multiple sclerosis.[18] Moreover, as we focused on selecting peptide markers present in multiple patients with CIDP, our workflow may have precluded the selection of CDR-peptides shared

by only a few patients. The large heterogeneity observed in CIDP could also further explain the presence of fewer CDR-peptides shared among patients.

In an independent validation cohort, we could validate the diagnostic performance of selected peptides SPSFQGQVTISADK and IVLTQSPATL through logistic regression models, albeit with reduced performance than was observed in the discovery cohort. A similar finding was noted for the multipeptide model which displayed a decreased AUC in the validation cohort. The challenge encountered in validating our selected POIs may be related to differences between cohorts. CIDP is characterized by a large heterogeneity and peptides or peptide panels suitable for diagnosing one cohort of CIDP patients may not be as useful for other cohorts. This is in line with earlier biomarker research in CIDP as initial findings regarding pathogenic antibodies directed against e.g. myelin components in CIDP could not be replicated by most later studies.[28] Patients with CIDP from the validation cohort also demonstrated increased disease duration compared to the discovery cohort as well as a larger spread in disease duration. It is possible that these patients, who have been suffering from the disease and subsequently been treated with immunomodulatory therapy for a longer period of time, display reduced disease activity and hence reduced levels of certain IgG-derived peptides. Moreover, the biological process of affinity maturation, in which somatic hypermutations can lead to the production of higher avidity antibodies, may lead to patients displaying more unique, higher avidity antibodies upon more extensive disease duration and hence prolonged antigen exposure. In turn, antibodies more widespread between individuals that originate from B-cells which have undergone less cycles of somatic hypermutation might be less abundantly present in these patients. Similar findings have been reported in studies on HIV-1 in which highly unique broadly neutralizing antibodies required years and multiple rounds of somatic hypermutation to develop in infected individuals.[29,30]

Another factor possibly affecting our results is the treatment of patients in our cohorts with immunomodulatory therapies, including IVIg therapy. None of the selected peptides were specifically upregulated in patients receiving immunomodulatory therapy and hence did not appear to originate from these therapies. However, the possibility that immunomodulating therapies may conversely result in decreased levels of certain IgG-derived peptides cannot be excluded, as proposed mechanisms of action of e.g. IVIg therapy include the provision of anti-idiotypic antibodies targeting autoantibodies and a suppression of autoantibody production.[31-33] Indeed, IVIg therapy has already demonstrated to decrease circulating autoantibody levels in various autoimmune diseases.[34,35] Hence, it may also result in decreased levels of peptides derived from circulating (auto-)antibodies. As our validation cohort included a higher proportion of patients treated with immunomodulatory therapies while displaying overall shorter treatment intervals, it is possible that treatment received by these patients may have affected our ability to validate selected peptides. Ideally, only samples of treatment naive and newly diagnosed patients would have been utilized. However, as we made use of samples prospectively collected from a clinical cohort of patients such samples were only sparsely available to us. Likewise, ideally, neurological controls should have been untreated and age- and sex-matched to the patients with CIDP but the limited number of samples from patients with these disorders available to us precluded us from doing so.

We also evaluated whether patients with CIDP of the discovery cohort could be clustered based on their IgG-derived peptide profile. Three clusters could be identified of 22, 16 and 6 patients, respectively. No difference in demographics could be determined between these three clusters. Nevertheless, we cannot exclude the possibility that the established clusters might differ in other features such as electrophysiological parameters.[23,36] Due to the clinical nature of our cohort, however, electrophysiological parameters were not available for most patients at time of sampling (often during follow-up, i.e. post-diagnosis) as nerve conduction studies were mostly

performed during diagnostic work-up. The clustering of patients with CIDP did also not relate to CIDP subtype. Nevertheless, it has been hypothesized that different CIDP variants might demonstrate different immunopathological mechanisms.[37] Theoretically, the IgG-derived peptide repertoire might therefore also differ between various subtypes. The relatively limited number of CIDP variants included in our cohort may have precluded the discovery of peptide profiles related to a certain CIDP variant. Likewise, it is possible that certain peptides might have shown increased diagnostic performance when considering CIDP subtypes as distinct entities in our analysis but the relatively limited number of patients with a CIDP variant precluded us from doing so. A follow-up study specifically focused on larger numbers of patients with typical CIDP and CIDP variants might reveal specific peptide profiles per variant and thereby further support the hypothesis of different variants displaying distinct immunopathological mechanisms.

The major limitation in current study is the use of a clinical cohort of patients resulting in significant variability in demographics both within and between cohorts, e.g. in disease duration, sex or number of patients treated with immunomodulatory therapy. Ideally, only newly diagnosed treatment naive patients and age- and sex-matched controls would have been included but as discussed earlier these were only available to us in very limited numbers. Nevertheless, despite this limitation, we demonstrated the antibody repertoire to be an interesting source of potential CIDP biomarkers. Hence, we encourage future prospective studies which might achieve greater success in finding and validating IgG-derived peptide biomarkers for CIDP by utilizing well-characterized treatment naive cohorts. Another remark can be made with respect to utilizing DDA for LC-MS/MS analysis of the validation cohort as opposed to utilizing targeted mass spectrometry. Once POIs were selected, we could have used the increased sensitivity and specificity of targeted mass spectrometry to measure these peptides in the validation cohort. However, as CIDP is a highly variable disease and we were forced to

utilize heterogeneous cohorts as discussed earlier, we acknowledged the risk of being unable to validate selected peptides. Hence, by opting for DDA also for analysis of the validation cohort, we generated a second peptide dataset, which can additionally be analyzed in later studies to identify other potentially interesting peptides. To circumvent the issue of lower abundant peptides not always being identified in DDA (as only the top N most abundant peptides eluting at a given timepoint are selected for fragmentation in this mode), we spiked QC samples of the validation cohort with an excess of synthetic version of POIs which ensured reliable identification of our targets in this cohort.

In conclusion, current study suggests differences to exist in serum IgG-derived peptides between patients with CIDP and controls, hence indicating them to be a potentially interesting source of CIDP biomarkers. Further prospective studies utilizing well-defined cohorts of treatment naive patients in an early disease stage are likely required to obtain validated IgG-derived peptide biomarkers for CIDP.

Study funding

This work was funded by the Catholic University of Leuven (KU Leuven) through C2-project Industrial Research Fund (IOF) financing (3M190242). J Godelaine has a PhD-fellowship of the Research Foundation – Flanders (FWO) (1191420N). K Poesen is a senior clinical investigator (18B2622N) of FWO and holds a Clinical Research fund of the University Hospitals Leuven. P Van Damme holds a senior clinical investigatorship of the Research Foundation - Flanders and is supported by the ALS Liga België and the KU Leuven funds “een hart voor ALS”, “Laeversfonds voor ALS Onderzoek” and the “Valéry Perrier Race against ALS Fund”.

Acknowledgments

The authors would like to thank all patients who contributed samples for use in this study as well as Mr. Kusay Arat from the KU Leuven SyBioMa mass spectrometry facility for his excellent technical assistance.

References

1. Broers MC, Bunschoten C, Nieboer D, Lingsma HF, Jacobs BC. Incidence and Prevalence of Chronic Inflammatory Demyelinating Polyradiculoneuropathy: A Systematic Review and Meta-Analysis. *Neuroepidemiology*. 2019;52(3-4):161-172. doi:10.1159/000494291

2. Van den Bergh PY, Hadden RD, Bouche P, et al. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of chronic inflammatory demyelinating polyradiculoneuropathy: report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society - first revision. *Eur J Neurol*. Mar 2010;17(3):356-63. doi:10.1111/j.1468-1331.2009.02930.x

3. Allen JA, Lewis RA. CIDP diagnostic pitfalls and perception of treatment benefit. *Neurology*. Aug 11 2015;85(6):498-504. doi:10.1212/WNL.0000000000001833

4. Brannagan TH, 3rd. Current diagnosis of CIDP: the need for biomarkers. *J Peripher Nerv Syst*. Jun 2011;16 Suppl 1:3-13. doi:10.1111/j.1529-8027.2011.00298.x

5. Mathey EK, Park SB, Hughes RA, et al. Chronic inflammatory demyelinating polyradiculoneuropathy: from pathology to phenotype. *J Neurol Neurosurg Psychiatry*. Sep 2015;86(9):973-85. doi:10.1136/jnnp-2014-309697

6. Pascual-Goni E, Martin-Aguilar L, Querol L. Autoantibodies in chronic inflammatory demyelinating polyradiculoneuropathy. *Curr Opin Neurol*. Oct 2019;32(5):651-657. doi:10.1097/WCO.0000000000000725

7. Allen JA, Merkies ISJ, Lewis RA. Monitoring Clinical Course and Treatment Response in Chronic Inflammatory Demyelinating Polyneuropathy During Routine Care: A Review of Clinical and Laboratory Assessment Measures. *JAMA Neurol*. Sep 1 2020;77(9):1159-1166. doi:10.1001/jamaneurol.2020.0781

8. Kwa MS, van Schaik IN, De Jonge RR, et al. Autoimmunoreactivity to Schwann cells in patients with inflammatory neuropathies. *Brain*. Feb 2003;126(Pt 2):361-75. doi:10.1093/brain/awg030

9. Gabriel CM, Gregson NA, Hughes RA. Anti-PMP22 antibodies in patients with inflammatory neuropathy. *J Neuroimmunol*. May 1 2000;104(2):139-46. doi:10.1016/s0165-5728(99)00269-6

10. Chen YT, Gure AO, Scanlan MJ. Serological analysis of expression cDNA libraries (SEREX): an immunoscreening technique for identifying immunogenic tumor antigens. *Methods Mol Med*. 2005;103:207-16.

11. de Costa D, Broodman I, Vanduijn MM, et al. Sequencing and quantifying IgG fragments and antigen-binding regions by mass spectrometry. *J Proteome Res*. Jun 4 2010;9(6):2937-45. doi:10.1021/pr901114w

12. Delves PJ, Roitt IM. *Encyclopedia of Immunology*. Academic Press; 1998.

13. Falkenburg WJJ, von Richthofen HJ, Rispens T. On the origin of rheumatoid factors: Insights from analyses of variable region sequences. *Semin Arthritis Rheum*. Jun 24 2018;doi:10.1016/j.semarthrit.2018.06.006

14. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. *J Allergy Clin Immunol*. Feb 2010;125(2 Suppl 2):S41-52. doi:10.1016/j.jaci.2009.09.046

15. Janeway CA Jr TP, Walport M, Shlomchik MJ. *Immunobiology: The Immune System in Health and Disease.* vol 5th edition. Garland Science; 2001.

16. de Costa D, Broodman I, Calame W, et al. Peptides from the variable region of specific antibodies are shared among lung cancer patients. *PLoS One*. 2014;9(5):e96029. doi:10.1371/journal.pone.0096029

17. Schmelter C, Perumal N, Funke S, Bell K, Pfeiffer N, Grus FH. Peptides of the variable IgG domain as potential biomarker candidates in primary open-angle glaucoma (POAG). *Hum Mol Genet*. Nov 15 2017;26(22):4451-4464. doi:10.1093/hmg/ddx332

18. Singh V, Stoop MP, Stingl C, et al. Cerebrospinal-fluid-derived immunoglobulin G of different multiple sclerosis patients shares mutated sequences in complementarity determining regions. *Mol Cell Proteomics*. Dec 2013;12(12):3924-34. doi:10.1074/mcp.M113.030346

19. Maat P, VanDuijn M, Brouwer E, et al. Mass spectrometric detection of antigen-specific immunoglobulin peptides in paraneoplastic patient sera. *J Autoimmun*. Jun 2012;38(4):354-60. doi:10.1016/j.jaut.2012.02.002

20. Lefranc MP, Giudicelli V, Ginestoux C, et al. IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res*. Jan 2009;37(Database issue):D1006-12. doi:10.1093/nar/gkn838

21. Ye J, Ma N, Madden TL, Ostell JM. IgBLAST: an immunoglobulin variable domain sequence analysis tool. *Nucleic Acids Res*. Jul 2013;41(Web Server issue):W34-40. doi:10.1093/nar/gkt382

22. Sun X-M, Yu X-P, Liu Y, Xu L, Di D-L. Combining bootstrap and uninformative variable elimination: Chemometric identification of metabonomic biomarkers by nonparametric analysis of discriminant partial least squares. *Chemometrics and Intelligent Laboratory Systems*. 2012/06/15/ 2012;115:37-43. doi:https://doi.org/10.1016/j.chemolab.2012.04.006

23. Moritz CP, Tholance Y, Stoevesandt O, Ferraud K, Camdessanché JP, Antoine JC. CIDP Antibodies Target Junction Proteins and Identify Patient Subgroups: An Autoantigenomic Approach. *Neurol Neuroimmunol Neuroinflamm*. Mar 4 2021;8(2)doi:10.1212/nxi.0000000000000944

24. Moritz CP, Stoevesandt O, Tholance Y, Camdessanché JP, Antoine JC. Proper definition of the set of autoantibody-targeted antigens relies on appropriate reference group selection. *N Biotechnol*. Jan 25 2021;60:168-172. doi:10.1016/j.nbt.2020.08.007

25. Zou H, Hastie T, Tibshirani R. Sparse Principal Component Analysis. *Journal of Computational and Graphical Statistics*. 2006/06/01 2006;15(2):265-286. doi:10.1198/106186006X113430

26. McCombe PA, Hardy TA, Nona RJ, Greer JM. Sex differences in Guillain Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy and experimental autoimmune neuritis. Review. *Frontiers in Immunology*. 2022-December-09 2022;13doi:10.3389/fimmu.2022.1038411

27. Hagen KM, Ousman SS. The immune response and aging in chronic inflammatory demyelinating polyradiculoneuropathy. *Journal of Neuroinflammation*. 2021/03/22 2021;18(1):78. doi:10.1186/s12974-021-02113-2

28. Querol L, Siles AM, Alba-Rovira R, et al. Antibodies against peripheral nerve antigens in chronic inflammatory demyelinating polyradiculoneuropathy. *Sci Rep*. Oct 31 2017;7(1):14411. doi:10.1038/s41598-017-14853-4

29. Mishra AK, Mariuzza RA. Insights into the Structural Basis of Antibody Affinity Maturation from Next-Generation Sequencing. *Front Immunol*. 2018;9:117. doi:10.3389/fimmu.2018.00117

30. Lee JH, Sutton HJ, Cottrell CA, et al. Long-primed germinal centres with enduring affinity maturation and clonal migration. *Nature*. 2022/09/01 2022;609(7929):998-1004. doi:10.1038/s41586-022-05216-9

31. Chaigne B, Mouthon L. Mechanisms of action of intravenous immunoglobulin. *Transfusion and Apheresis Science*. 2017/02/01/ 2017;56(1):45-49. doi:https://doi.org/10.1016/j.transci.2016.12.017

32. Ruzhansky K, Brannagan TH, 3rd. Intravenous immunoglobulin for treatment of neuromuscular disease. *Neurol Clin Pract*. Oct 2013;3(5):440-445. doi:10.1212/CPJ.0b013e3182a78ecf

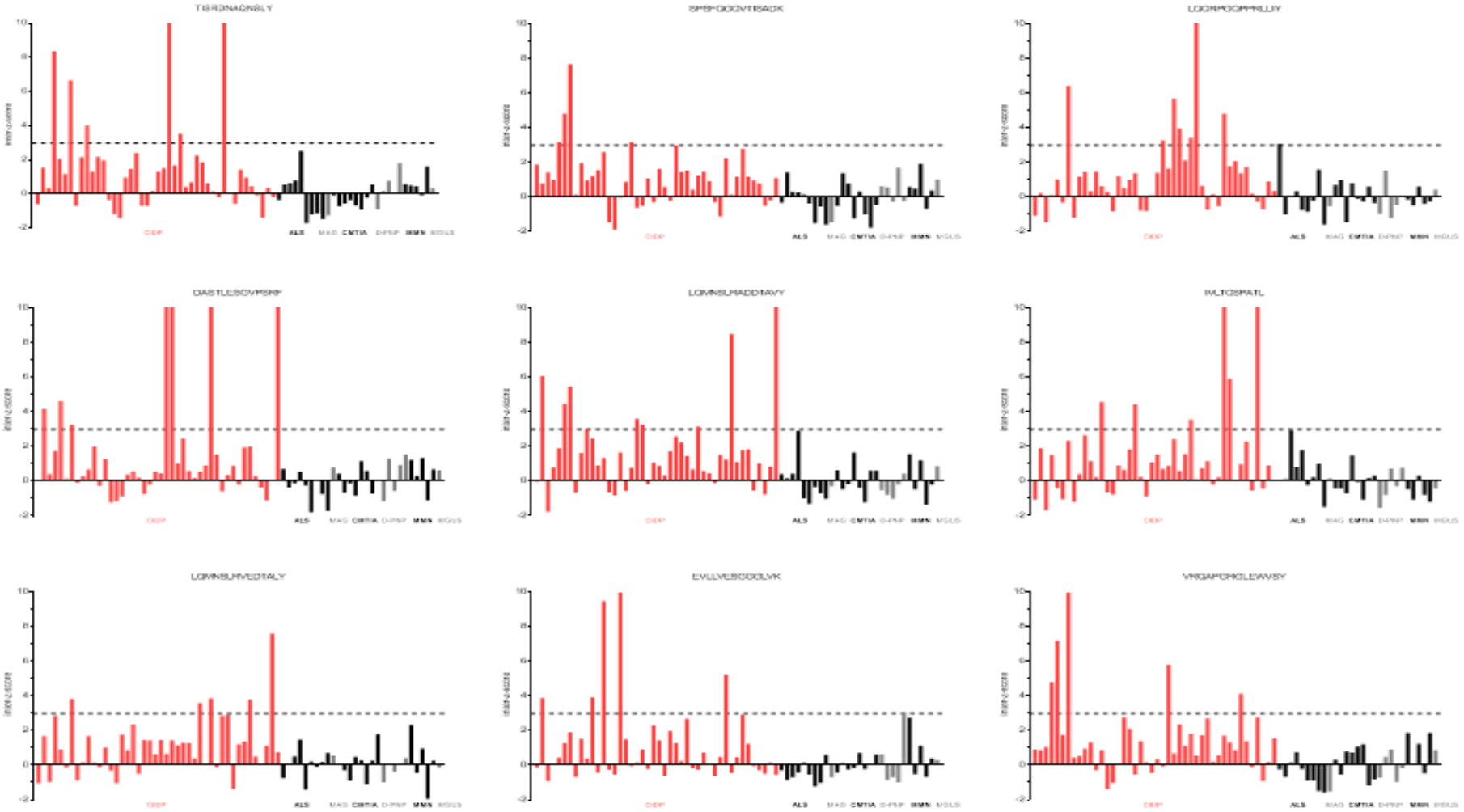
33. Dalakas MC. IgG4-Mediated Neurologic Autoimmunities: Understanding the Pathogenicity of IgG4, Ineffectiveness of IVIg, and Long-Lasting Benefits of Anti-B Cell Therapies. *Neurol Neuroimmunol Neuroinflamm*. Jan 2022;9(1)doi:10.1212/nxi.0000000000001116

34. Dalakas MC. Intravenous immunoglobulin in autoimmune neuromuscular diseases. *Jama*. May 19 2004;291(19):2367-75. doi:10.1001/jama.291.19.2367

35. Fujita A, Ogata H, Yamasaki R, Matsushita T, Kira JI. Parallel fluctuation of anti-neurofascin 155 antibody levels with clinico-electrophysiological findings in patients with chronic inflammatory demyelinating polyradiculoneuropathy. *J Neurol Sci*. Jan 15 2018;384:107-112. doi:10.1016/j.jns.2017.11.035

36. Baek SH, Hong YH, Choi SJ, et al. Electrodiagnostic data-driven clustering identifies a prognostically different subgroup of patients with chronic inflammatory demyelinating polyneuropathy. *J Neurol Neurosurg Psychiatry*. Jun 2019;90(6):674-680. doi:10.1136/jnnp-2018-319758

37. Ikeda S, Koike H, Nishi R, et al. Clinicopathological characteristics of subtypes of chronic inflammatory demyelinating polyradiculoneuropathy. *J Neurol Neurosurg Psychiatry*. Sep 2019;90(9):988-996. doi:10.1136/jnnp-2019-320741

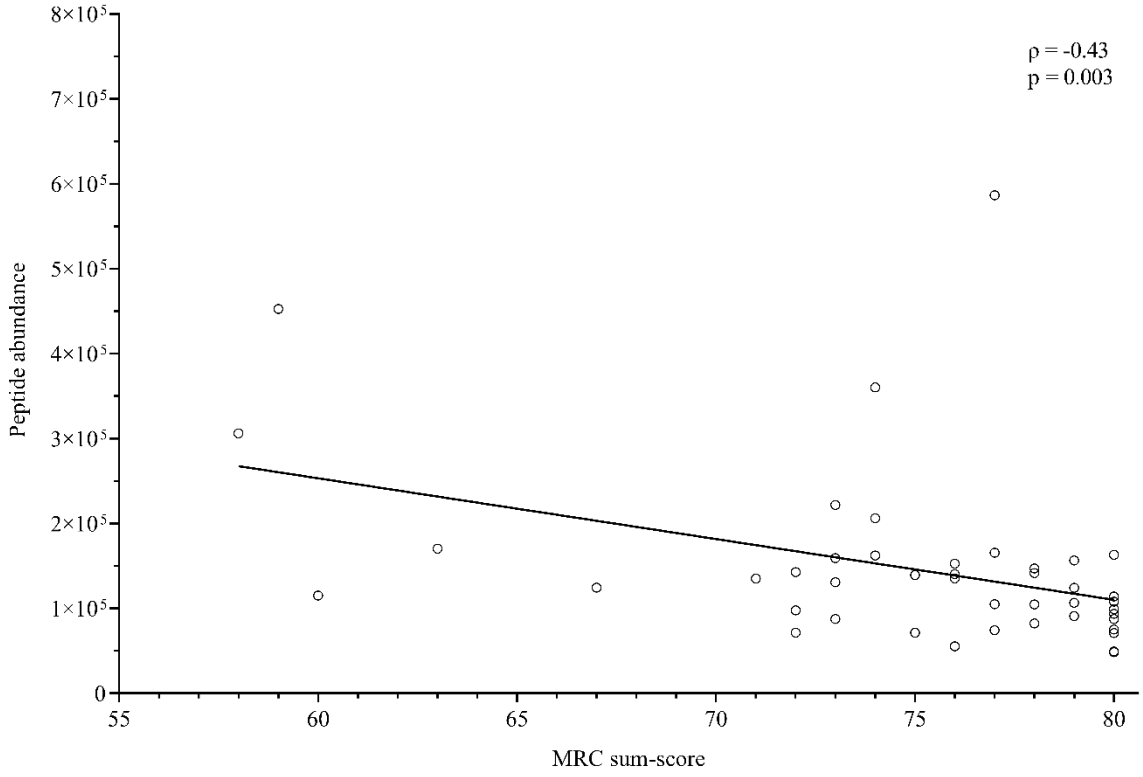


eFigure 1. Inter-z-score profiles in the discovery cohort of selected peptides of interest.

Inter-z-scores are calculated based on the standard deviation and mean of the abundance observed in disease controls. Patients with CIDP are depicted in red, neurological controls in black and grey. The dotted line represents the 3SD cut-off.

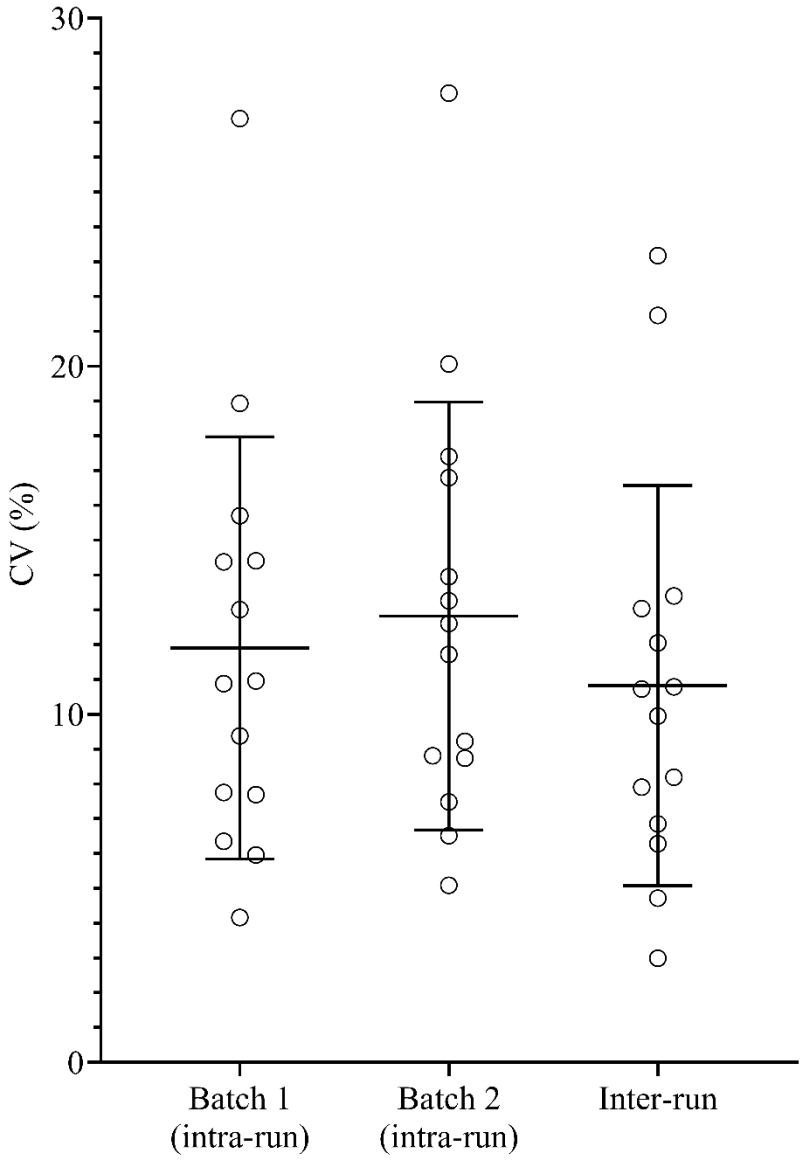
eFigure 1. Inter-z-score profiles in the discovery cohort of selected peptides of interest (continued).

Inter-z-scores are calculated based on the standard deviation and mean of the abundance observed in disease controls. Patients with CIDP are depicted in red, neurological controls in black and grey. The dotted line represents the 3SD cut-off.



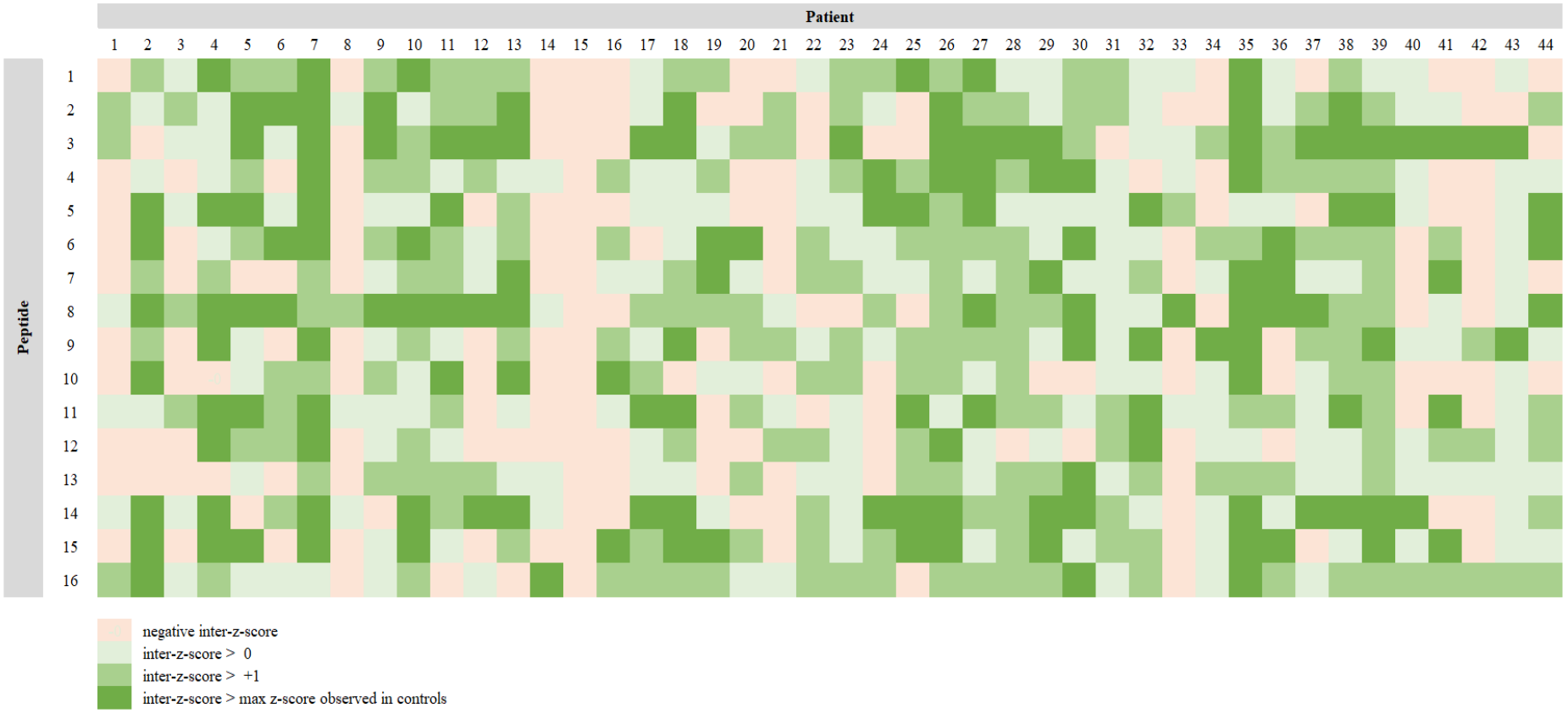
eFigure 2. Correlation between the MRC sum-score and the abundance of peptide TISRDNAQNSLY.

Abbreviations: MRC, Medical Research Council.



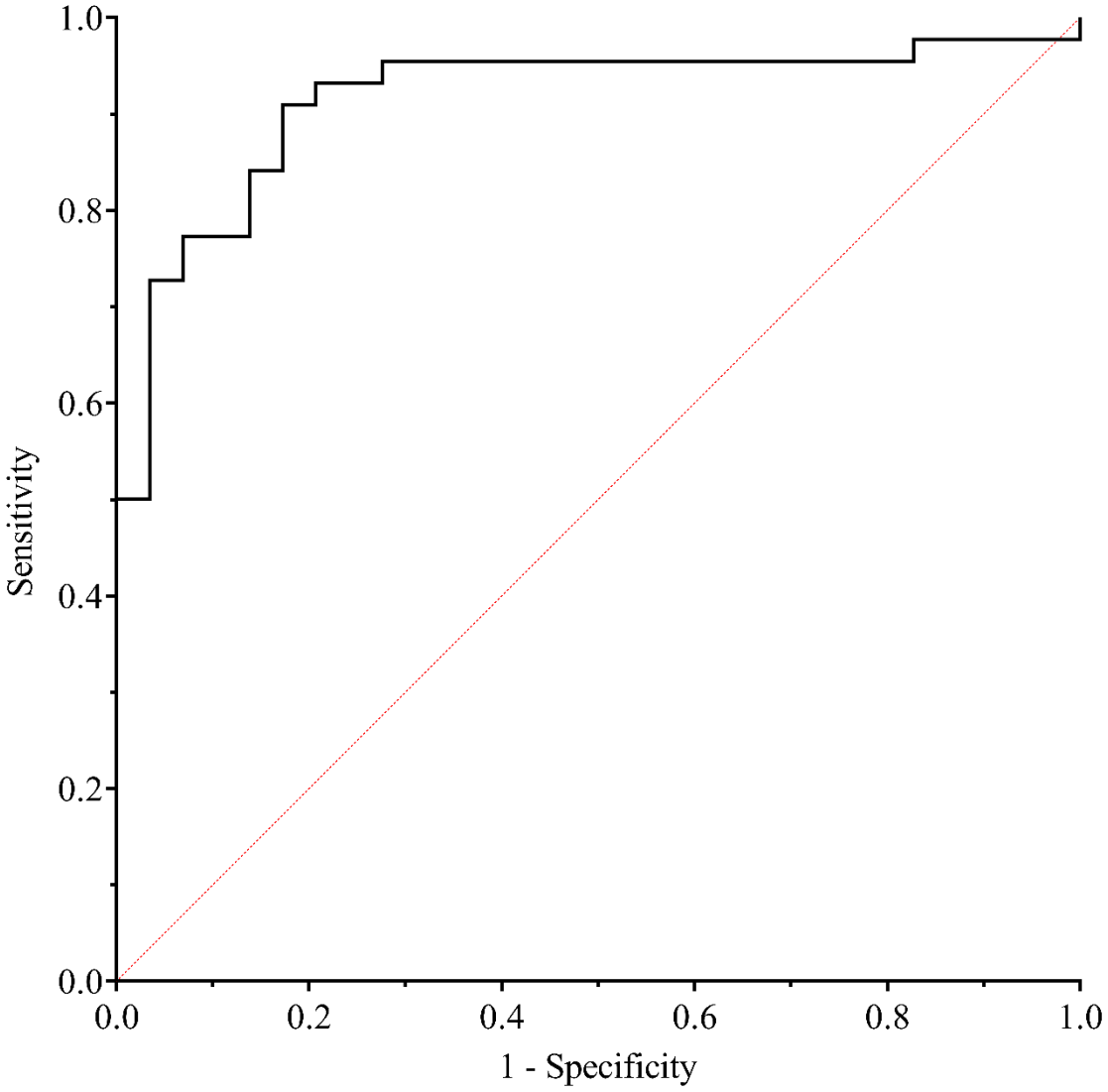
eFigure 3. Intra- and inter-run CVs observed for the selected peptides of interest.

The reproducibility of measuring the selected peptides was evaluated through the sample preparation and LC-MS/MS analysis of 2x6 replicates (eMethods). Each bullet represents a selected peptide. Mean and SD are presented on the figure.



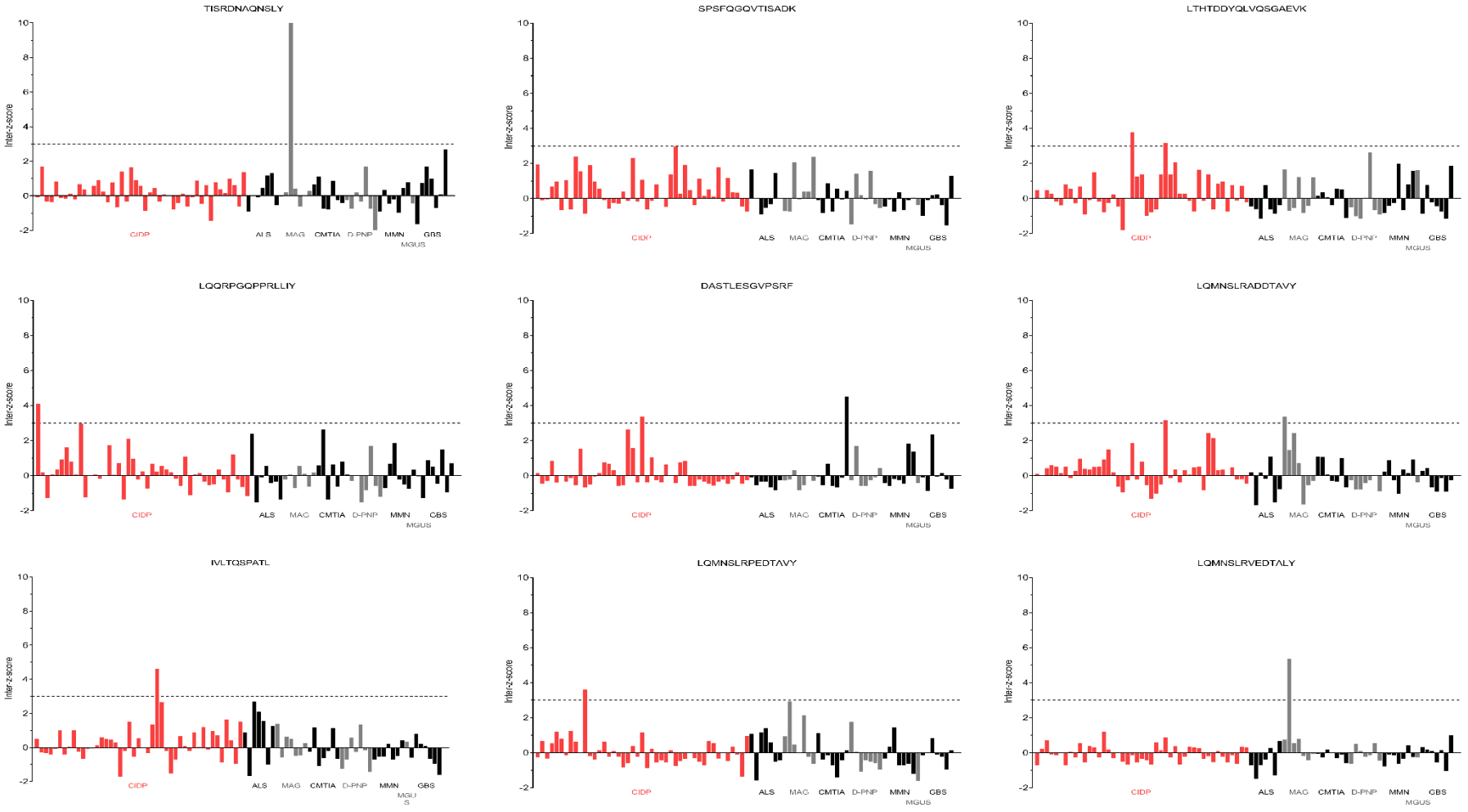
eFigure 4. Heatmap of the inter-z-scores for selected peptides of interest for patients with CIDP of the discovery cohort.

Patients identified at a fixed specificity of 100% per peptide (i.e. patients with an inter-z-score above the maximum inter-z-score observed in controls for that peptide) are marked in dark green. At 100% specificity, combining the four and six most promising POIs allows identification of 33 (75%) and 35 (79.5%) of patients with CIDP, respectively. Taken together, all 16 POIs could identify 37/44 (84.9%) of patients with CIDP at 100% specificity.



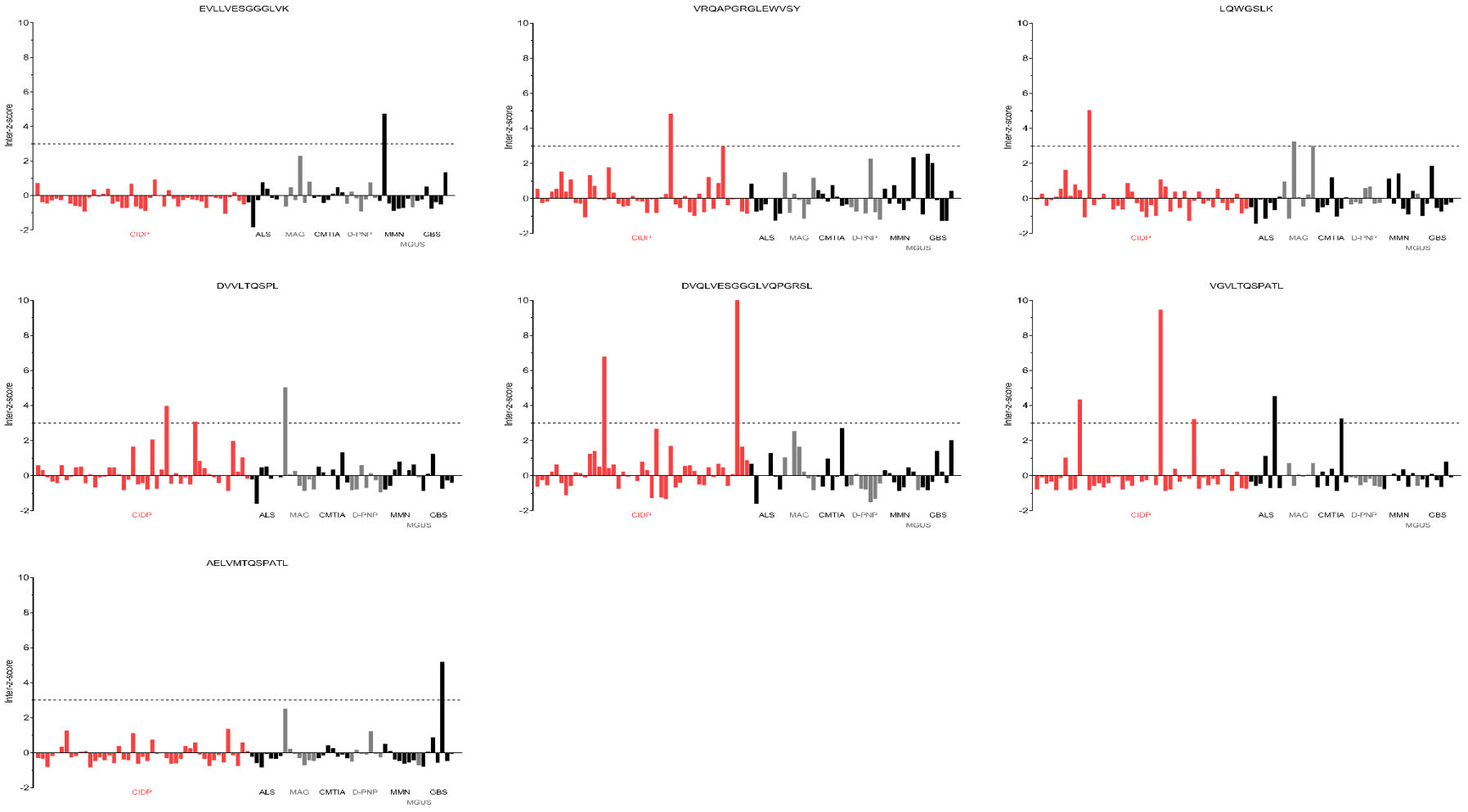
eFigure 5. ROC-curve of the five-peptide model applied to the discovery cohort.

Based on stepwise multiple logistic regression, five of the 16 selected peptides were incorporated into a multipeptide diagnostic model. This model demonstrated a Youden index-based AUC of 91.5% (95% CI 84.7% to 98.4%, $p = < 0.001$) in the discovery cohort.



eFigure 6. Inter-z-score profiles in the validation cohort of selected peptides of interest.

Inter-z-scores are calculated based on the standard deviation and mean of the abundance observed in disease controls. Patients with CIDP are depicted in red, neurological controls in black and grey. The dotted line represents the 3SD cut-off.



eFigure 6. Inter-z-score profiles in the validation cohort of selected peptides of interest (continued).

Inter-z-scores are calculated based on the standard deviation and mean of the abundance observed in disease controls. Patients with CIDP are depicted in red, neurological controls in black and grey. The dotted line represents the 3SD cut-off.

Table 1. Demographics of the study population.

	Discovery cohort			Validation cohort			Between cohorts
	CIDP	Controls	*p*	CIDP	Controls	*p*	*p*
Included, n (%)		29 (39.7)			43 (48.9)		
Of which ALS		*9*			*7*		
CMTIA		*8*			*7*		
D-PNP	44 (60.3)	*5*	-	45 (51.1)	*7*	-	-
MAG		*1*			*7*		
MGUS		*1*			*1*		
MMN		*5*			*7*		
GBS		*0*			*7*		
Age, years Mean (SD)	63.8 (11.0)	56.4 (17.2)	**0.029**	63.0 (13.4)	59.8 (17.1)	0.324	0.789
Sex, male/female	37/7	15/14	**0.004**	38/7	18/25	**<0.001**	0.318
Receiving immunomodulatory therapy, n (%) *Of which IVIg*	32 (72.7) *29*	7 (24.1) *6*	**<0.001**	37 (82.2) *33*	13 (30.2) *13*	**<0.001**	0.751
Time since last dose of IVIg, days Median (IQR), range	56 (28), 25-127	42 (7), 35-84	0.356	41 (23), 14-88	46 (50), 17-147	0.051	0.054
Disease duration, years Mean (SD)	10.9 (6.6)	-	-	18.3 (30.1)	-	-	**0.043**
INCAT score Median (IQR), range	3 (2), 0-8	-	-	3 (2), 0-10	-	-	0.960
MRC sum-score Median (IQR), range	77 (6), 58-80	-	-	75 (8), 0-80	-	-	0.455
EFNS/PNS 2010, n (%)							
Definite	44 (100)	-	-	44 (97.8)	-	-	1.000
Probable	0			1 (2.2)			
CIDP phenotype, n (%)							
Typical	31 (70.5)	-	-	33 (73.3)	-	-	0.816
CIDP variant	13 (29.5)[a]			12 (26.7)[b]			

[a] CIDP variants: 4x distal CIDP, 6x multifocal CIDP, 3x sensory CIDP. [b] CIDP variants: 7x distal CIDP, 3x multifocal CIDP, 1x motor CIDP, 1x sensory CIDP. Abbreviations: ALS, amyotrophic lateral sclerosis; CMTIA, Charcot-Marie-Tooth IA; D-PNP, diabetic peripheral neuropathy; EFNS/PNS, European Federation of Neurological Societies/Peripheral Nerve Society; GBS, Guillain-Barré syndrome; INCAT, Inflammatory Neuropathy Cause and Treatment scale; IVIg, intravenous immunoglobulin; MAG, anti-myelin-associated glycoprotein-related neuropathy; MGUS, monoclonal gammopathy of unknown significance – related neuropathy; MMN, multifocal motor neuropathy; MRC, Medical Research Council

Table 2. Characteristics of selected peptides

Peptide	Sequence	m/z	charge	Protein description (accession number | origin)	CDR/ FR[a]	Blast bit score	IMGT % identified	*p*
1	TISRDNAQNSLY	691.339	2	P01782 | Ig heavy chain variable region	FR3	27.7	100%	0.003
2	SPSFQGQVTISADK	732.870	2	A0A0C4DH38 | Ig heavy chain variable region	FR3	31.2	100%	0.001
3	LTHTDDYQLVQSGAEVK	635.313	3	A0A0G2JMI3 | Ig heavy chain variable region	FR1	36.6	84.2%	<0.001
4	LQQRPGQPPRLLIY	560.329	3	A0A0C4DH68 | Ig kappa variable region	FR2	33.5	100%	0.004
5	DASTLESGVPSRF	683.342	2	A0A0B4J2D9 | Ig kappa variable region	CDR2-FR3	27.7	92.3%	0.036
6	LQMNSLRADDTAVY	799.379	2	P0DTE1 | Ig heavy chain variable region	FR3	29.6	92.9%	<0.001
7	IVLTQSPATL	521.810	2	A0A0C4DH25 | Ig kappa variable region	FR1	22.3	100%	0.004
8	LQMNSLRPEDTAVY	818.911	2	P0DTE1 | Ig heavy chain variable region	FR3	28.9	85.70%	<0.001
9	LQMNSLRVEDTALY	827.412	2	A0A0C4DH32 | Ig heavy chain variable region	FR3	29.6	92.9%	0.001
10	EVLLVESGGGLVK	650.380	2	A0A0B4J1V0 | Ig heavy chain variable region	FR1	25.8	92.30%	0.005
11	VRQAPGRGLEWVSY	539.954	3	P01763 | Ig heavy chain variable region	FR2	33.1	92.9%	<0.001
12	LQWGSLK	416.240	2	A0A0J9YXX1 | Ig heavy chain variable region	FR3	17.3	85.70%	0.035
13	DVVLTQSPL	486.274	2	P06310 | Ig kappa variable region	FR1	20.4	88.9%	<0.001
14	DVQLVESGGGLVQPGRSL	905.984	2	P01782 | Ig heavy chain variable region	FR1	37.4	94.40%	<0.001
15	VGVLTQSPATL	543.314	2	P04433 | Ig kappa variable region	FR1	20.8	100%	0.005
16	AELVMTQSPATL	630.829	2	P01624 | Ig kappa variable region	FR1	23.9	90.90%	<0.001

Underlined amino acids were aligned to a CDR-region. [a] the position of the sequence in the Ig structure is indicated. Abbreviations: CDR, complementary-determining region; FR, framework region; Ig, immunoglobulin.

Table 3. Logistic regression models of selected peptides in the discovery cohort.

Peptide[a]	Univariate model				Multivariable model[c]		
	OR	95% CI	*p*	AUC (*p*)	OR	95% CI	*p*
1	1.212	1.067 to 1.418	**<0.001**	0.707 (**0.003**)	1.186	1.048 to 1.393	**0.021**
2	1.112	1.044 to 1.200	**<0.001**	0.731 (**<0.001**)	1.094	1.024 to 1.185	**0.016**
3	1.920	1.358 to 2.903	**<0.001**	0.75 (**<0.001**)	1.921	1.313 to 3.052	**0.002**
4	1.543	1.147 to 2.243	**0.002**	0.701 (**0.004**)	1.445	1.044 to 2.189	0.053
5	1.533	1.110 to 2.345	**0.002**	0.646 (**0.036**)	1.487	1.062 to 2.325	0.057
6	1.735	1.287 to 2.504	**<0.001**	0.777 (**<0.001**)	1.869	1.337 to 2.811	**<0.001**
7	2.743	1.345 to 6.654	**0.002**	0.698 (**0.004**)	2.691	1.218 to 7.028	**0.028**
8	2.234	1.494 to 3.714	**<0.001**	0.789 (**<0.001**)	2.154	1.401 to 3.687	**0.002**
9	1.988	1.324 to 3.278	**<0.001**	0.722 (**0.001**)	1.980	1.292 to 3.397	**0.005**
10	3.116	1.305 to 9.821	**0.004**	0.692 (**0.006**)	4.771	1.511 to 20.354	**0.018**
11	3.671	1.752 to 9.028	**<0.001**	0.745 (**<0.001**)	3.501	1.599 to 9.434	**0.005**
12[b]	1.292	1.050 to 1.680	**0.013**	0.647 (**0.035**)	1.253	1.012 to 1.642	0.065
13[b]	1.389	1.107 to 1.879	**0.001**	0.755 (**<0.001**)	1.404	1.118 to 1.919	**0.012**
14	1.842	1.334 to 2.766	**<0.001**	0.765 (**<0.001**)	1.791	1.265 to 2.768	**0.003**
15[b]	1.716	1.197 to 2.695	**<0.001**	0.694 (**0.005**)	1.726	1.162 to 2.840	**0.016**
16[b]	2.497	1.577 to 4.416	**<0.001**	0.795 (**<0.001**)	2.410	1.481 to 4.434	**0.002**

[a] For identity of the peptides, we refer to Table 2 of the main manuscript. Odds ratios are reported per 10 000 increase in normalized abundances unless otherwise specified. [b] Odds ratios reported per 1000 increase in normalized abundance (lower abundant peptides). [c] Full multivariable logistic regression models are available in eTable 2.

Table 4. Logistic regression models of selected peptides in the validation cohort

Peptide[a]	Univariate model				Multivariable model[c]		
	OR	95% CI	*p*	AUC (*p*)	OR	95% CI	*p*
1	0.997	0.970 to 1.002	0.314	0.562 (0.314)	0.996	0.972 to 1.002	0.383
2	1.017	1.003 to 1.033	**0.017**	0.632 (**0.033**)	1.014	0.999 to 1.031	0.078
3	1.031	0.994 to 1.072	0.108	0.62 (0.052)	1.040	0.998 to 1.089	0.073
4	1.049	0.960 to 1.154	0.291	0.572 (0.244)	1.032	0.936 to 1.147	0.535
5	1.015	0.957 to 1.081	0.618	0.563 (0.307)	1.086	0.984 to 1.208	0.113
6	1.070	0.980 to 1.179	0.133	0.622 (**0.049**)	1.296	1.036 to 1.672	**0.033**
7	1.132	0.948 to 1.376	0.174	0.597 (0.118)	1.008	0.945 to 1.079	0.815
8	1.010	0.953 to 1.072	0.732	0.546 (0.455)	0.976	0.899 to 1.052	0.503
9	0.987	0.913 to 1.059	0.706	0.513 (0.831)	0.942	0.853 to 1.013	0.159
10	0.944	0.863 to 1.012	0.111	0.562 (0.319)	1.054	0.915 to 1.235	0.488
11	1.054	0.929 to 1.209	0.418	0.558 (0.352)	0.983	0.916 to 1.055	0.611
12[b]	1.003	0.940 to 1.073	0.915	0.528 (0.649)	1.014	0.998 to 1.033	0.106
13[b]	1.008	0.994 to 1.024	0.261	0.571 (0.255)	1.069	0.986 to 1.200	0.198
14	1.056	0.990 to 1.161	0.110	0.598 (0.112)	1.058	0.474 to 2.755	0.894
15[b]	1.070	0.501 to 2.414	0.855	0.581 (0.193)	0.810	0.499 to 1.197	0.308
16[b]	0.891	0.584 to 1.277	0.527	0.504 (0.950)	1.023	0.958 to 1.096	0.495

[a] For identity of the peptides, we refer to Table 2 of the main manuscript. Odds ratios are reported per 10 000 increase in normalized abundances unless otherwise specified. [b] Odds ratios reported per 1000 increase in normalized abundance (lower abundant peptides). [c] Full multivariable logistic regression models are available in eTable 4.

eMethods

LC-MS/MS analysis

Five microliters of desalted peptides (dissolved in 50 µL 0.1% formic acid/5% acetonitrile) were injected and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with an Acclaim PepMap100 pre-column (C18 3 µm-100 Å, Thermo Scientific) and a C18 PepMap RSLC (2 µm, 50 µm-15cm, Thermo Scientific) using a gradient (300 nL/min) of 0–4% buffer B (80% acetonitrile, 0.08% FA) for 3 min, 4–10% B for 7 min, 10–35% B for 25 min, 35–38% B for 5 min, 38-40% B for 2 min, 40-65% B for 5 min, 65 – 95% B for 1 min, 95% B for 9 min, 95%-5% B for 1 min and 5% B for 9 min. The Orbitrap Elite Velos Pro ETD mass spectrometer (Thermo Scientific) was operated in positive ion mode with a nanospray voltage of 2.1 kV and a source temperature of 275 °C. Pierce LTQ Velos ESI positive ion calibration mix (88323, Thermo Scientific) was used as an external calibrant. The instrument was operated in data-dependent acquisition mode with a survey MS scan at a resolution of 60,000 for the mass range of m/z 375–1500 for precursor ions, followed by MS/MS scans of the top 20 most intense peaks with +2, +3, +4, and above charged ions above a minimum signal threshold count of 500 at rapid resolution setting of ion trap using normalized collision energy of 35 eV with an isolation window of 2.0 m/z and dynamic exclusion of 30 s. All data were acquired with Xcalibur 3.0.63.3 software (Thermo Scientific)

Determination and confirmation of peptide sequences

Targeted mass spectrometry (parallel reaction monitoring (PRM)) was used to elucidate amino acid sequences of peptides of interest (POIs) selected from the DDA-analysis of the discovery cohort. First, samples showing highest abundance for selected POIs were pooled and next prepared in the same way as for the DDA LC-MS/MS runs. Next, five microliters of desalted

peptides were injected and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with an Acclaim PepMap 100 pre-column (C18 3 μm–100 Å, Thermo Scientific) and a C18 PepMap RSLC (2 μm, 50 μm-15 cm, Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). For LC, the same gradient was used as described above for the DDA-analysis. The Q Exactive Orbitrap mass spectrometer (Thermo Scientific) was operated in positive ion mode with a nanospray voltage of 1.8 kV and a source temperature of 250 °C. Pierce LTQ Velos ESI positive ion calibration mix (88323, Thermo Scientific) was used as an external calibrant. To avoid overlapping time windows, the samples were analyzed on the Q Exactive in PRM mode in two separate runs. All data were acquired with Xcalibur 3.1.66.10 software (Thermo Scientific).

Possible amino acid sequences for POIs identified with the highest confidence by Mascot and/or PEAKS were retained and synthetic peptides with amino acid sequence identical to the elucidated sequence ordered (Thermo Scientific). Synthetic peptides were pooled and diluted in 0.1% formic acid/ 5% acetonitrile so that 1ng per synthetic peptide was injected and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with an Acclaim PepMap 100 pre-column (C18 3 μm–100 Å, Thermo Scientific) and a C18 PepMap RSLC (2 μm, 50 μm-15 cm, Thermo Scientific) coupled to the Q Exactive Orbitrap mass spectrometer (Thermo Scientific) operating in data-dependent acquisition mode. Mass, charge, retention time and mass spectra of the synthetic peptides were compared to the same parameters as obtained for selected POIs to confirm the amino acid sequence that was elucidated by Mascot or PEAKS after PRM.

Evaluating the reproducibility of measuring selected peptides

To evaluate reproducibility of both sample preparation and LC-MS/MS for the measurement of peptides of interest selected in the discovery cohort, one designated patient sample was selected and divided into 12 identical aliquots. Each aliquot was subsequently prepared as an individual sample according to our standard workflow (IgG isolation, digestion, desalting). Next, the prepared replicates were analyzed on the Orbitrap Elite mass spectrometer in two batches of six samples using identical settings as described for the DDA-analysis above. The two batches were measured two weeks apart from each other with other samples not related to this study measured in the meantime, to also evaluate stability of the LC-MS/MS measurements over time. To quantify reproducibility, both intra-run CV's (within one batch of replicates) as well as inter-run CV's (between the two batches) were calculated for each peptide of interest.

Construction of a multipeptide diagnostic model

A multipeptide model was constructed to determine whether improved diagnostic performance could be obtained for such a model compared to individual IgG-derived peptides. Stepwise logistic regression was performed on the peptides selected in the discovery cohort, trained on this discovery cohort and subsequently tested on the validation cohort. Stepwise variable selection was performed in the logistic regression model on the basis of Akaike information criterion. To correct for different peptide abundances measured in the two cohorts, which impeded us from applying a model established in one cohort directly to the other cohort, peptide abundances in each cohort were first normalized by applying a normalization factor to each cohort in which an equal average peptide abundance was assumed per subject. 95% confidence intervals for AUC of the multipeptide model in both the discovery and validation cohort was calculated by DeLong method of computation.

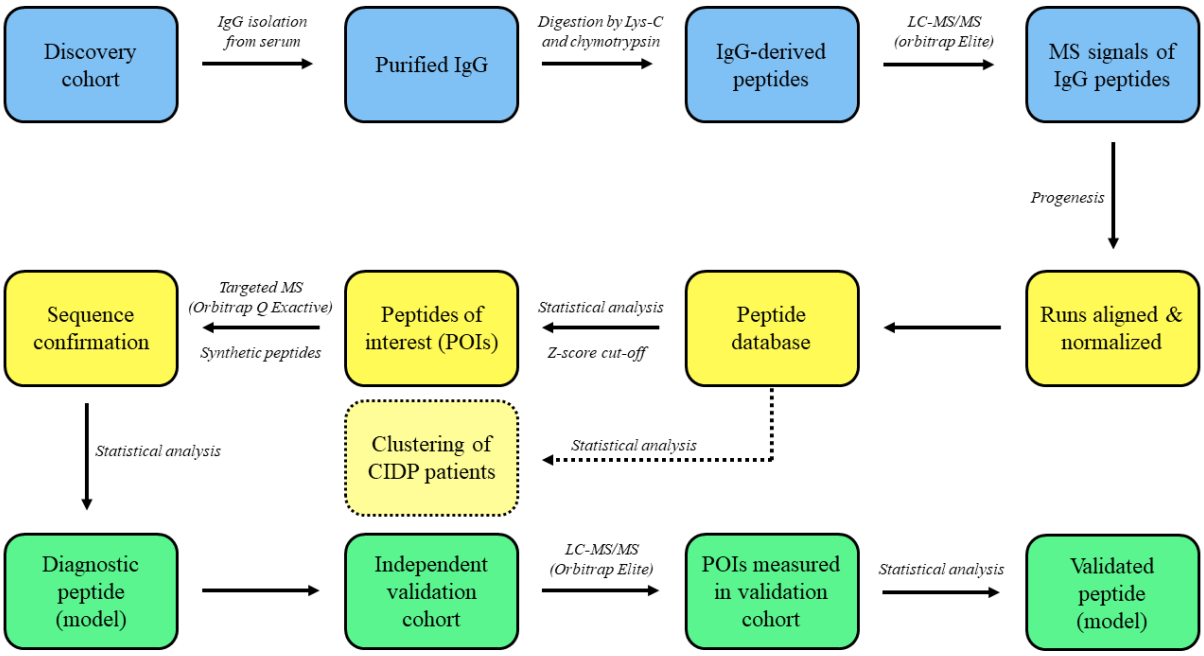


Figure 1. Flowchart of the methodology and study design.

IgG-derived peptides were studied in serum of patients and controls by means of Orbitrap LC-MS/MS. First, statistical analysis was performed on IgG-derived peptide data and potential peptides of interest (POIs) were selected in the discovery cohort after which the sequence of these POIs was determined via targeted mass spectrometry and confirmed against synthetic peptides. Next, diagnostic performance of individual POIs as well as of a multipeptide model incorporating the POIs was evaluated in the discovery cohort and subsequently reassessed in an independent validation cohort (primary objective). IgG-derived peptides were also studied as measures to objectively cluster patients with CIDP into clinical subgroups (secondary objective).

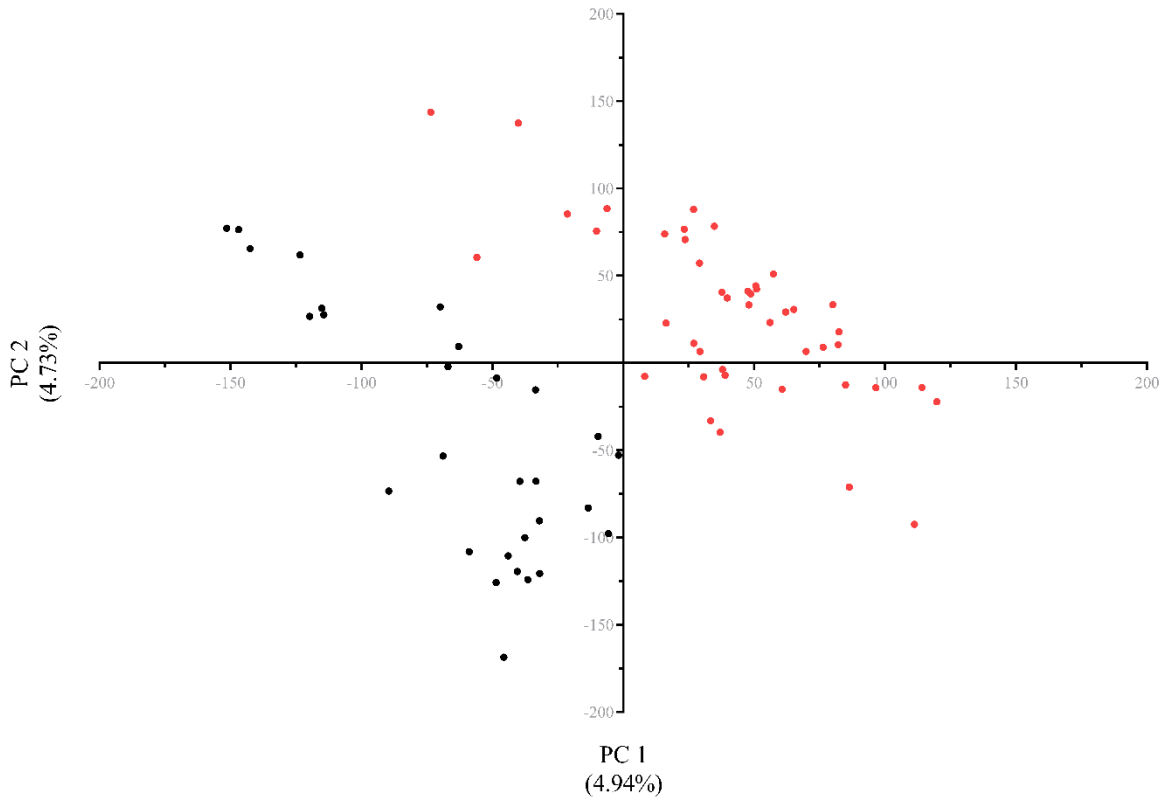


Figure 2. Score plot of the partial least-squares discriminant analysis.

Score plot of the first two components of a partial least-squares discriminant analysis with CIDP versus controls as dependent variable and peptide abundances as independent variables. Brackets indicate proportion of variance explained by the components. Patients with CIDP are depicted by red bullets, neurological controls by black bullets.

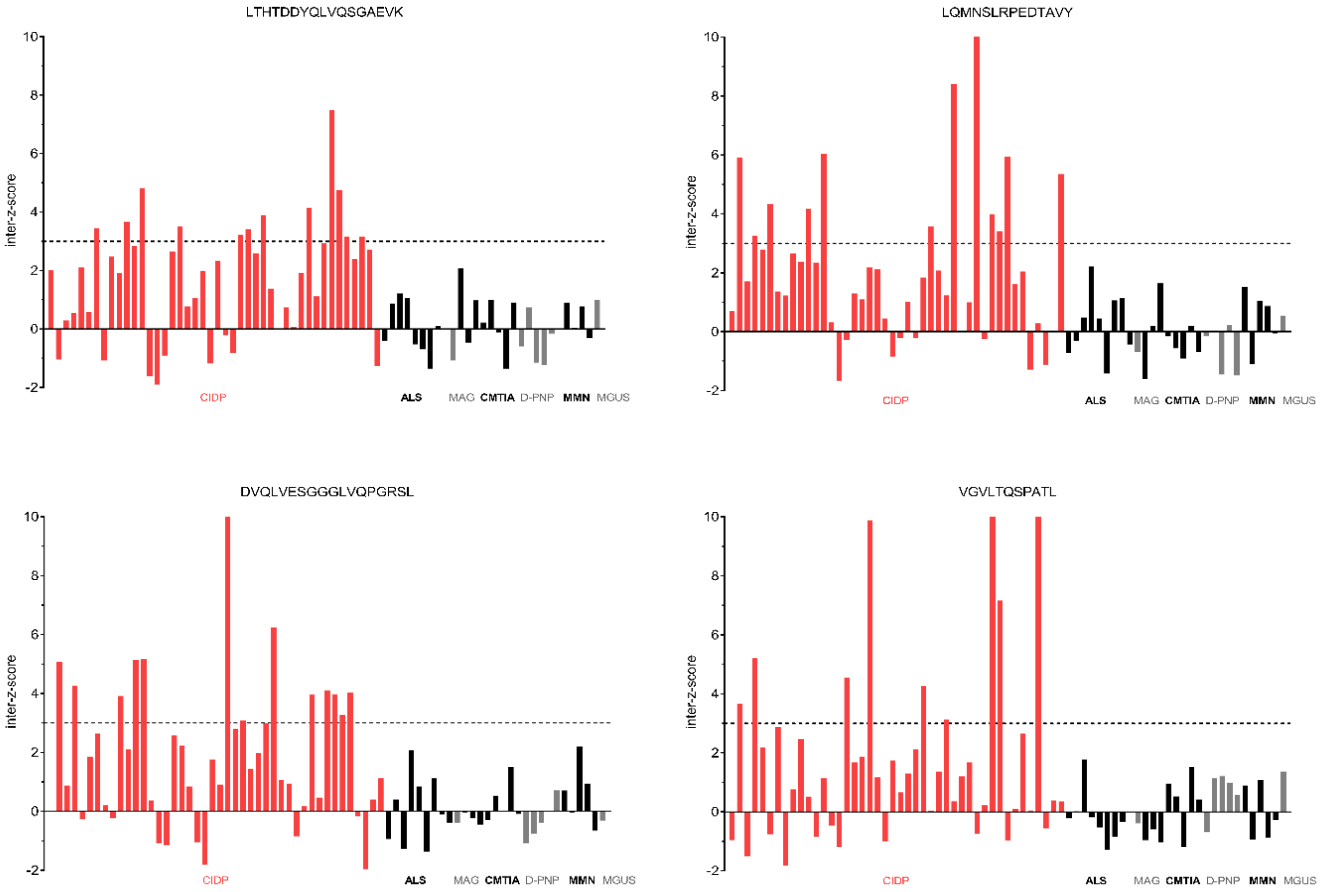


Fig 3. Discovery cohort inter-z-score profile of four selected peptides of interest.

Inter-z-scores represent the number of standard deviations the peptide abundance differed from the mean peptide abundance measured in neurological controls. The four peptides out of 16 retained peptides that showed an inter-z-score of +3SD for most of the patients with CIDP are depicted here. For each peptide, the sequence is depicted above the graph. Inter-z-score profiles of the other 12 selected peptides are shown in eFigure 1. Patients with CIDP are depicted in red, neurological controls in black and grey. The dotted line represents the 3SD cut-off.