eMethods

Discovery and validation 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 ions 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 (targeted-selected ion monitoring-dd/MS2 (tSIM-dd/MS2)) was used to elucidate possible 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 prepared in the same way as for the discovery 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 was operated in positive ion mode with a nanospray voltage of 2.1 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. For tSIM-dd/MS2 settings, the threshold ion count was 1e+5 at 35000 resolution, a maximum IT of 59 msec and an isolation window of 3 m/z. The include list for tSIM contained m/z, charge and retention time (±2 minutes) values for POIs as specified in Table 2.

Tandem mass spectra obtained via tSIM-dd/MS2 analysis were next 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 10 ppm; fragment mass tolerance of 0.02 Da; a maximum of four missed cleavages; Lys-C + chymotrypsin 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). Sequences identified with the highest confidence by Mascot and/or PEAKS were retained as potential amino acid sequence for the POIs.

Next, to confirm the final amino acid sequence of POIs, synthetic peptides (Thermo Scientific) with sequence identical to the potential sequences for POIs (i.e. identified by Mascot and/or PEAKS with the highest confidence) were ordered. The synthetic peptides were pooled and

diluted in 0.1% formic acid/ 5% acetonitrile so that 1ng of each synthetic peptide was injected into the Q Exactive Orbitrap mass spectrometer operating in DDA mode with a survey MS scan at a resolution of 70,000 (FWHM at m/z 200) for the mass range of m/z 400–1600 for precursor ions, followed by MS/MS scans of the top ten most intense ions with +2, +3, +4, and + 5 charged ions above a threshold ion count of 1e+6 at 17 500 resolution using normalized collision energy of 25 eV with an isolation window of 3.0 m/z, Apex trigger of 5-15 seconds and dynamic exclusion of 10 seconds. All data were acquired with Xcalibur 3.1.66.10 software (Thermo Scientific). Mass, charge, retention time and mass spectra of the measured synthetic peptides were then compared to the same parameters as for selected POIs to confirm amino acid sequences suggested earlier by Mascot and/or PEAKS as the correct sequence for the POI.

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 were calculated by DeLong method of computation.