Isotopic N,N-dimethyl leucine tags for absolute quantification of clusterin and apolipoprotein E in blood samples from Alzheimer's disease

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Abstract: Alzheimer's disease (AD) is one of the top leading causes of death in the United States. In the past decades, great efforts have been put into biomarker discovery for early diagnosis and treatment of AD. Here, this study aims to quantitative analysis clusterin (CLU) and apolipoprotein E (APOE) in blood samples from AD patients and evaluate these two proteins as potential biomarkers in AD diagnosis. In-house-synthesized 5-plex isotopic *N,N*-dimethyl leucine (iDiLeu) tags are used to label target peptide standards with multiple channels. We found that the levels of

CLU and APOE showed clear differences between male AD and female AD groups but not in the male and female non-AD groups. In contrast, the levels of serum CLU and APOE did not show statistically significant differences in the AD groups and non-AD groups. Dissecting CLU and APOE heterogeneity in AD pathogenesis may therefore enable grading the pathological relevance for sex-related pathways, guiding the way to personalized medicine.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia among the elders, which is characterized by the formation of amyloid-β deposition and aggregation of protein tau in the brain.[1] In the past decades, tremendous efforts have been made to understand underlying pathogenesis, identify protective and pathogenic genes, and discover and validate imaging, cerebrospinal fluid (CSF) and blood-based biomarkers. Imaging biomarkers such as magnetic resonance imaging (MRI), ^{18}fluorodeoxyglucose (^{18}FDG) positron emission tomography (PET), and amyloid-PET are well-validated for AD diagnosis.[2] In addition, CSF biomarkers including amyloid β1–42 (Aβ42), amyloid β1–40 (Aβ40), phosphorylated tau181 (p-tau181), p-tau217 and total tau (t-tau) are well-established.[3, 4] Compared to imaging biomarkers and CSF-based biomarker, blood-based biomarkers enable easier access and can reduce the cost and invasiveness in clinical practice. Therefore, great effort has been made to discover and validate blood-based biomarkers for AD. Thus far, plasma amyloid β 42/40 ratio (Aβ42/40) has been reported to show reduction in AD and can be sensitively measured as biomarker.[5-7] Also, recent studies show excellent performance of phosphorylated tau 181 and 217 in plasma as diagnostic biomarkers for AD versus other dementias.[8-10] The encouraging and rapid development in blood-based assays is promising for prescreening in clinical trials with reduced cost and invasiveness, and once validated, for clinical

diagnosis.[11] In addition to Aβ and tau, numerous other proteins have also been investigated to discover other potential biomarkers and to facilitate the understanding of AD pathology. Among these proteins, both clusterin (CLU) and apolipoprotein E (APOE) are related to Aβ clearance and deposition. [12-15]

CLU is a 78-80 kDa heterodimeric glycoprotein that is highly expressed in the central nervous system (CNS) and functions as an extracellular chaperone that can facilitate the transport of Aβ across the blood-brain barrier.[16-18] It has been suggested that CLU can bind to Aβ oligomers and interfere with peptide aggregation and inhibit the growth of extracellular amyloid fibrils.[12-14] There is also significant positive correlation between the concentration of CLU and the regional levels of insoluble Aβ42 in brain regions.[12-14] APOE is a 299-amino acid protein with a molecular mass of ~34 kDa that serves important functions in lipoprotein-mediated lipid transportation between organs.[19] The peripheral pool of APOE is mainly expressed by the liver, and in the CNS, APOE is mainly produced by astrocytes and microglia.[20, 21] Previous study showed that APOE may negatively affect clearance of soluble Aβ and Aβ deposition at different stages of plaque formation.[15]

However, comparing APOE concentrations in patients at different developmental stages of AD showed different and unconvincing results in CSF and plasma samples in different studies.[22-25] Previous studies in our lab observed significant dysregulation of APOE expression levels in CSF of AD patients compared to healthy control.[26, 27] Therefore, it would be meaningful to evaluate the concentrations of these two proteins in blood sample to see whether they can serve as potential biomarkers in AD. Enzyme-linked immunosorbent assay (ELISA) is commonly used for measuring protein expression levels in blood and clinical biomarker verification, but the requirement of high-quality antibodies and difficulties when it comes to developing multiplexed

assays limit its applicability to quantify novel protein biomarkers.[28] In contrast, absolute quantification (AQUA) strategies using synthetic heavy isotope-encoded peptides as internal standard can be applied to quantifying targeted peptides in the blood samples.[29] However, the high cost of the isotopically labeled internal peptides limits its usage for verification of large number of candidate biomarkers. Alternatively, strategies by chemically introducing heavy isotopes into target peptides to produce different precursor ion masses in MS1 spectra or reporter ions in tandem MS (MS/MS) spectra can be used for relative and absolute quantification, such as mTRAQ, iTRAQ and TMT.[30-34] The development of 5-plex isotopic N,N-dimethyl leucine (iDiLeu) tags enables cost-effective and improved quantification throughput as compared to other absolute quantification methods mentioned above.[35] iDiLeu-labeling strategy enables construction of a 4-point calibration curve and has been adopted previously to quantify analytes in CSF samples.[26] In this strategy, we applied a 5-plex iDiLeu-tagging strategy to do absolute quantification for APOE and CLU in serum samples of AD and non-AD participants (**Figure 1**) and investigate their potential to serve as blood biomarkers for AD.

2. Materials and methods

2.1. Serum samples

Serum samples were collected from 10 patients with AD (5 men and 5 women), and 9 non-AD individuals (5 men and 4 women). Table 1 summarizes the demographic characteristics of the participants. CSF samples were collected by lumbar puncture. Blood was sampled by venipuncture at the same time as the CSF and collected in gel separator tubes. Following clotting for 30 min, the sample was centrifuged at 2200 x g for 10 min with the temperature set at +20 °C. The supernatant was collected, aliquoted and stored at -80 °C until analysis. The samples were from

patients who sought medical advice because of cognitive impairment. Patients were designated as AD and non-AD according to CSF biomarker levels using cutoffs that are >90% specific for AD: total-tau (T-tau) >350 pg/mL, phospho-tau 181 (P-tau181) >60 pg/mL and Aβ42 <530 pg/mL (2 out 3 positive).[36] None of the biochemically normal subjects fulfilled these criteria. The study was approved by the regional ethics committee at the University of Gothenburg.

Table 1. Characteristics of the current study participants.

Diagnosis	Gender	Age (years)	CSF Aβ42 (pg/ml)	CSF T-tau	CSF p-tau181
Non-AD, n = 5	M	69.2 ± 8.7	810 ± 159	242 ± 75	35 ± 9
AD, n = 5	M	75.0 ± 7.4	391 ± 63	853 ± 168	105 ± 19
Non-AD, n = 5	F	70.0 ± 4.3	765 ± 117	226 ± 84	33 ± 11
AD, n = 4	F	66.2 ± 7.4	470 ± 112	732 ± 168	88 ± 13

Data are presented as mean ± standard deviation. AD, Alzheimer's disease; M, male; F, female; Aβ42, amyloid-β42; T-tau, total tau protein; p-tau181, phosphorylated tau181.

2.2 Serum protein digestion

Ten µL of serum was diluted in 190 µL water and protein concentrations were measured by protein assay kit (Thermo Scientific Pierce) following the manufacturer's protocols. Serum proteins were dissolved in 6 M urea, 50 mM tris buffer (contains 5 mM $CaCl_2$, 20 mM NaCl, and 1 tablet of EDTA-free protease inhibitor cocktail, pH 8), reduced by dithiothreitol at a final concentration of 5 mM for 1 h. Fifteen mM of iodoacetamide was added for alkylation of cysteines by incubating

for 30 min in dark, followed by addition of 5 mM dithiothreitol for quenching unreacted iodoacetamide. Then the protein mixture was digested with trypsin/LysC (Thermo Scientific, Rockford, IL, United States) at a protein:enzyme ratio of 25:1 at 37 °C for 3 h. The sample was then diluted by 50 mM Tris buffer to a final urea concentration of 1 M, followed by incubation at 37 °C for 15 h. The digestion reaction was quenched by acidification with 10% trifluoroacetic acid (TFA) to a final concentration of 0.3% (pH 3), followed by desalting with Sep-Pak C18 cartridges (Waters Corporation, Milford, Massachusetts, MA, United States). Then the digested peptides were dried down with a SpeedVac concentrator (Thermo Scientific, Waltham, MA, United States) and resuspended in 0.1% formic acid (FA). Peptide concentration was measured by peptide assay (Thermo Scientific, Rockford, IL, United States). Then peptide digestion was aliquot at 50 µg in each tube and was dried in vacuo for further use.

2.3 Peptide and protein standards preparation

Stock solutions of synthetic human peptide (Biomatik, Ontario, Canada) ASSIIDELFQDR (unique peptide of CLU), were prepared at 1 mM. APOE protein standard was digested according to digestion procedure described above. iDiLeu reagents were in-house synthesized as previously described.[35, 37] In each peptide aliquot, 4 nmol of CLU peptide standard (about 4 µg) was combined with 400 pmol APOE protein digests (about 14.5 µg), and the mixture was resuspended in 10 µL of 0.5 M triethylammonium bicarbonate (TEAB) buffer. Each mixture was labeled by each iDiLeu tag (d0, d3, d6, d9, and d12) at a ratio of 10:1 (tags−peptide, by weight) or higher with excessive tags. A volume of 40 µL of activated iDiLeu tags was added to each aliquot to 80:20 organic−aqueous solution ratio and shake for 1.5 h at room temperature, respectively. Hydroxylamine (50%) was added to a final concentration of 0.25% to quench the reaction.

Unreacted reagents and by-products of iDiLeu-labeling reaction were removed by SCX TopTips (PolyLC INC., Columbia, MD, United States) according to the manufacturer's protocol. The eluate was dried in vacuo and desalted with Bond Elut OMIX C18 pipette tips (Agilent Technologies, Santa Clara, CA, United States).

Serum protein digests were reconstituted in 10 μL of 0.5 M TEAB and labeled with d0 iDiLeu tag separately according to the peptide standards labeling procedure described above. The other four channels labeled peptide standards were spiked into each d0-labeled serum sample in a ratio of 1:5:25:100. The combined sample was cleaned up with SCX TopTips and desalted with Bond Elut OMIX C18 pipette tips. For correction factor calculation, four μg of each labeled peptide standard was cleaned up with SCX TopTips and desalted with Bond Elut OMIX C18 pipette tips. All the labeled samples were then dried in vacuo and reconstituted in 50 uL of 0.1% formic acid (FA) in water.

2.4 NanoLC−MS acquisition

LC separation was performed on a Dionex Ultimate 3000 nanoLC system (Thermo Scientific). Capillary column (15 cm length, 75 μm i.d.) was self-fabricated and packed with Bridged Ethylene Hybrid C18 materials (1.7 μm, 130 Å, Waters Corporation). Samples were loaded onto the column in 97% solvent A (water, 0.1% FA) at a flow rate of 0.3 μL/min. Reverse-phase separation was performed using a linear gradient from 3% to 35% solvent B (ACN, 0.1% FA) for 90 min. Peptide elutions were electrosprayed into an Orbitrap Fusion Lumos Tribrid quadrupole-ion trap-Orbitrap mass spectrometer (Thermo Scientific). Full MS scan was acquired in profile-mode ranging from *m/z* 350 to 950 at a resolution of 60 K. Automatic gain control (AGC) target was set as 1×10^6, and maximum injection time was 120 ms. The top 20 most abundant precursor ions were selected for

stepped higher-energy collisional dissociation fragmentation with a dynamic exclusion for 40 s with 10 ppm. MS^2 spectra were acquired at centroid mode. The resolution was set as 30 K, isolation window of 1.6 Th, stepped normalized collision energy (NCE) of 27, 30 and 33, the maximum injection time of 60 ms, AGC target of 1×10^5, and fixed first mass of *m/z* 110. Each sample was acquired in technical triplicates.

2.5 Data analysis

Protein identification was performed using MaxQuant (version 1.5.2.8) against SwissProt human database (February 2020) with 1% false discovery rate (FDR) at peptide and protein level. The first search peptide tolerance for precursor and product ion were 20 ppm and 0.02 Da, respectively. The maximum missed cleavages per peptide was 2. Fixed modification was set as carbamidomethylation of cysteine residues (+57.0215 Da) and d0 labeling of N-terminal of peptides and lysine residues (+141.1154 Da). Oxidation of methionine (+15.9949 Da) was selected as variable modification. The intensity obtained from relative quantification in MaxQuant was further analyzed in Excel for advanced downstream analysis. For absolute quantification of CLU and APOE, protein identification was performed on Peaks Studio Xpro software (Bioinformatics Solutions, Inc., Waterloo, ON, Canada). The data refinement was used to adjust precursor mass by default. All the raw files were searched with UniProt *Homo sapiens* reviewed database with trypsin as digestion enzyme. The mass error tolerance for precursor ions was 15 ppm using monoisotopic mass and using 0.02 Da for product ions. The maximum missed cleavages per peptide was two, allowed to be cleaved at both ends of the peptides. Fixed modification was set as carbamidomethylation of cysteine residues (57.0215 Da), iDiLeu labels (+141.1154 Da, +144.1313 Da, +147.1409 Da, +150.1631 Da, and +153.1644 Da for d0, d3, d6, d9 and d12

respectively) of peptide N-termini and lysine residues, along with oxidation of methionine (+15.9949 Da) chosen as variable modifications. Peptides with FDR < 1% were unambiguous identification. Peak areas generated by Genesis peak detection algorithm in Thermo Xcalibur 4.0 software were used for absolute quantification. The precursor ion integration tolerance was 0.015 Da. Retention time of extracted ion chromatogram of 5-plex iDiLeu-labeled peptides was required to be within 2 min. Isotopic interference correction factors were applied to each sample to rectify the raw values. A two-sided Student t-test was used to evaluate the differences of the protein expression levels in the serum samples. A p-value of 0.05 or less is the cutoff for significance.

3. Results and discussion

3.1 Quantitative performance of 5-Plex iDiLeu-labeling strategy

At the beginning, label-free serum protein digests were tested in LC-MS/MS and CLU and APOE were identified with confidence. A mixture of 5-plex iDiLeu-labeled peptide standard/protein standard digests were first used to evaluate the performance of the labeling strategy. Impurities of reagents incorporated during iDiLeu synthesis can compromise the quantification accuracy. When 5-plex iDiLeu reagents label peptides, mass additions of 141.1154, 144.1313, 147.1409, 150.1631, and 153.1644 Da are added into peptides by d0, d3, d6, d9, and d12 labels, respectively (**Supplementary Table S1**). The mass of five monoisotopic precursors will be 3 Da or 6 Da apart which will cause isotopic interference. To overcome these pitfalls, we firstly determined the correction factors for each of the 5-plex iDiLeu tags to ensure accurate quantification (**Supplementary Table S2**). More details about how to determine the correction factors can be found in previous literature.[35] To determine the linear concentration range of this iDiLeu-labeling strategy, unique peptides standards from CLU and APOE proteins were labeled with 5-plex iDiLeu

tags and combined at a ratio of 10:1:5:25:100. The reason why we chose these two peptides as internal standards was that our preliminary results showed that these peptides could generate good MS/MS spectra for confident identification based on the data analysis in Maxquant. Also, previous studies also tried these peptides.[26, 38] The concentration of stock solution of iDiLeu-labeled CLU peptide (ASSIIDELFQDR) ranged from 50 ~ 5000 fmol/μL and then diluted to prepare concentrations of 10 ~ 1000 fmol/μL and 2 ~ 200 fmol/μL. For the APOE peptides, the concentration of stock solution of iDiLeu-labeled APOE peptide (AATVGSLAGQPLQER) ranged from 5 ~ 500 fmol/μL and then diluted twice to prepare concentrations of 1 ~ 100 fmol/μL and 0.2 ~ 20 fmol/μL. Based on literature, these concentration ranges should cover all the reported concentrations of these two proteins in human serum samples.[25, 39] **Figure 2** displayed peak areas of iDiLeu-labeled CLU peptides and APOE peptides as a function of concentrations across LC–MS analysis in duplicates by normalization to d12 channel (the normalized peak area of d12 channel was set as 100). Each calibration curve revealed a good correlation with coefficient above 0.99 and a linear concentration range spanning two orders of magnitude. For the concentration range used for quantification of CLU in serum, the coefficient of variation (CV) was below 6.5% in the range of 10 ~ 1000 fmol/μL. The CV was below 5% in the concentration of 1 ~ 100 fmol/μL for APOE. The high linearity in the concentration range we used for quantification confirmed that iDiLeu-tagging is a good strategy for absolute quantification of these two proteins.

3.2 Absolute quantification of CLU and APOE in Alzheimer's disease

The quantitative performance of 5-plex iDiLeu-labeling strategy has been evaluated with high accuracy and linearity, allowing for confident absolute quantification of CLU and APOE in serum samples. Here, we used undepleted serum samples because CLU and APOE have relatively high

abundance and can be detected by the sensitive instrument platform. Compared with antibody-based protein depletion strategy, which may lead to co-immunoprecipitation and sample loss, the undepleted serum samples may give a more accurate quantification with lower variation.[40] Instead of using MS1 scan only, we set up the data-dependent acquisition to do absolute quantification for two peptides of CLU and APOE using 5-plex iDiLeu reagents, together with relative quantification for global proteome in both AD participants and non-AD participants. Each serum sample was labeled with d0 channel, and standard peptides with a ratio of 1:5:25:100 were labeled with d3, d6, d9, d12, respectively. The labeled peptide standards were then spiked into each serum sample with equal amount at the same ratio. The final concentration of labeled peptide standard ASSIIDELFQDR ranged from 0.5 ~ 50 pmol/50 μg serum protein digests and the final concentration of labeled APOE digests ranged from 0.05 ~ 5 pmol/50 μg serum protein digests. iDiLeu-labeled peptides were fragmented under stepped NCE of 27, 30 and 33. **Figure 3** shows representative tandem mass spectra of d0-labeled ASSIIDELFQDR and AATVGSLAGQPLQER in serum sample. iDiLeu d0 tag was fragmented to produce dimethylated immonium reporter ion at *m/z* of 114.1. Rich b- and y- product ions were matched to specific peptide sequence with false discovery rate (FDR) < 1%, which reflects the statistical significance of the peptide-spectrum match.[41] The plot of normalized peak area of each peptide against concentration displayed high linearity, which ensured the accuracy of protein quantification (**Figure 4A and 4C**). A standard deviation within 12% was demonstrated across the triplicates run in each sample, indicating a good reproducibility within the technical replicates.

The average concentrations of CLU were quantified as 287 and 379 μg/mL in male and female of AD, and 299 and 283 μg/mL in serum of male and female of non-AD, respectively (**Figure 4B**). The average concentrations of APOE were 29 and 53 μg/mL in male and female of AD, and 31

and 35 μg/mL in male and female of AD, respectively (**Figure 4D**). From the data we got, we found that there were no significant differences of APOE or CLU between AD and non-AD participants of different genders. However, the tendency was consistent with our previous discovery for the concentration level of APOE in CSF of preclinical AD and healthy control groups, where higher APOE concentration was observed in AD compared with non-AD of female participants, where lower APOE concentration was observed in AD compared with non-AD of male participants.[26] Interestingly, the data showed a higher expression level of both CLU and APOE in the male AD participants compared with female AD participants, with a p-value of 0.034 and 0.033 respectively. This is consistent with our previous study showing that the APOE level in female AD patients was higher than that in CSF of male AD patients.[26] This may be because that female and male are impacted by some of the AD risk factors differently and thus have different expression levels of AD-related protiens.[42] Principal component analysis with CLU and APOE showed some separation between the AD and non-AD participants (**Supplemental Figure 2**). Previous studies show that the isoforms of APOE and post-translational modification of APOE are related to AD risks.[20] Further studies working on characterize the isoforms and post-translational modifications of CLU and APOE may help illustrate their roles in AD pathogenesis.

For the relative quantification, we found the differences of the summed intensity from sample to sample could exceed 50%, which may result from variance of sample loss during SCX cleanup for removing labeling reagents and variance of column and instrument performance. Considering the large differences between samples, we didn't further process the data for relative quantification. Given the labeled iDiLeu peptide standards were mixed with the labeled samples, variances occurred during following sample preparation and data acquisition would not affect the accuracy

of the absolute quantification. The low standard deviation within triplicate technical runs validates the robustness of this strategy for absolute quantification.

4. Conclusion

In summary, we measured the concentration of CLU and APOE in human serum using custom-developed 5-plex iDiLeu tags. Although we did not find significant expression levels between the AD participants and non-AD participants, we observed different expression levels of these two proteins between female and male of the AD participants. If replicated, these results might provide new insights into the underlying pathogenesis of AD and possibly inspire development of therapeutic approaches. In this study, we showed the robustness of iDiLeu in absolute quantification of proteins using Orbitrap mass spectrometer, and this strategy could also be easily adapted on other instrument platforms such as a time-of-flight (TOF) mass spectrometer. A test for the accuracy and reproducibility of the iDiLeu-based absolute quantification on TOF MS instruments would be very meaningful, and the advantage of the high speed of TOF MS instrument may enable the quantification of a large sample cohort and facilitate more conclusive results. This strategy could be applied to protein quantification and biomarker verification across a broad biofluids and various diseases.

5. References

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Figures

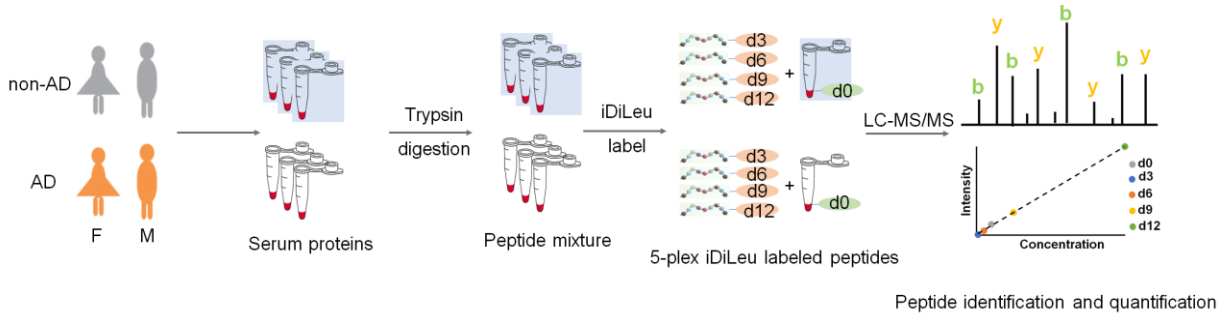

Figure 1. Workflow for absolute protein quantification using iDiLeu-labeling strategy.

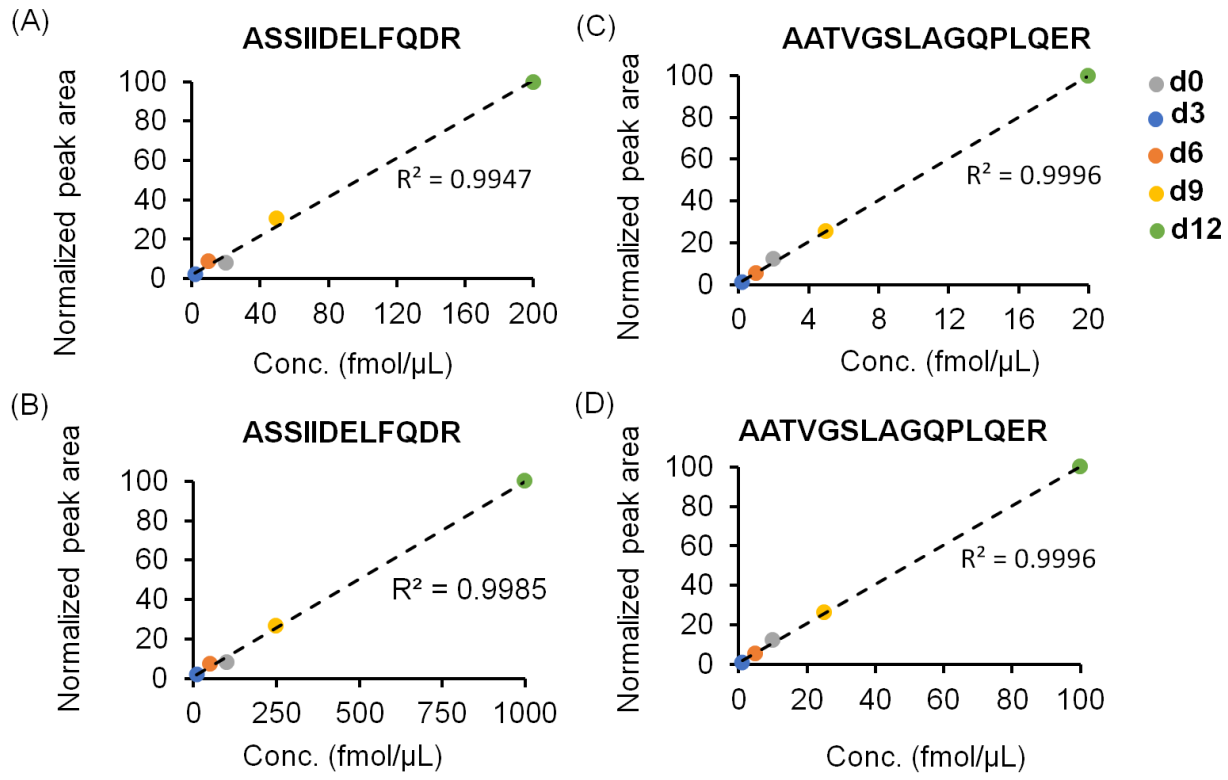

Figure 2. Calibration curves of 5-plex iDiLeu-labeled CLU peptide and APOE peptide. The normalized peak areas of precursor ions were plotted as a function of CLU peptide concentrations at 2–200 fmol/μL (A), 10–1000 fmol/μL (B). The normalized peak areas of precursor ions were plotted as a function of APOE peptide concentrations at 0.20–20 fmol/μL (C), 10–1000 fmol/μL (D). Two μL was injected for each LC-MS/MS run. ASSIIDELFQD is the peptide of CLU and AATVGSLAGQPLQER is the peptide of APOE.

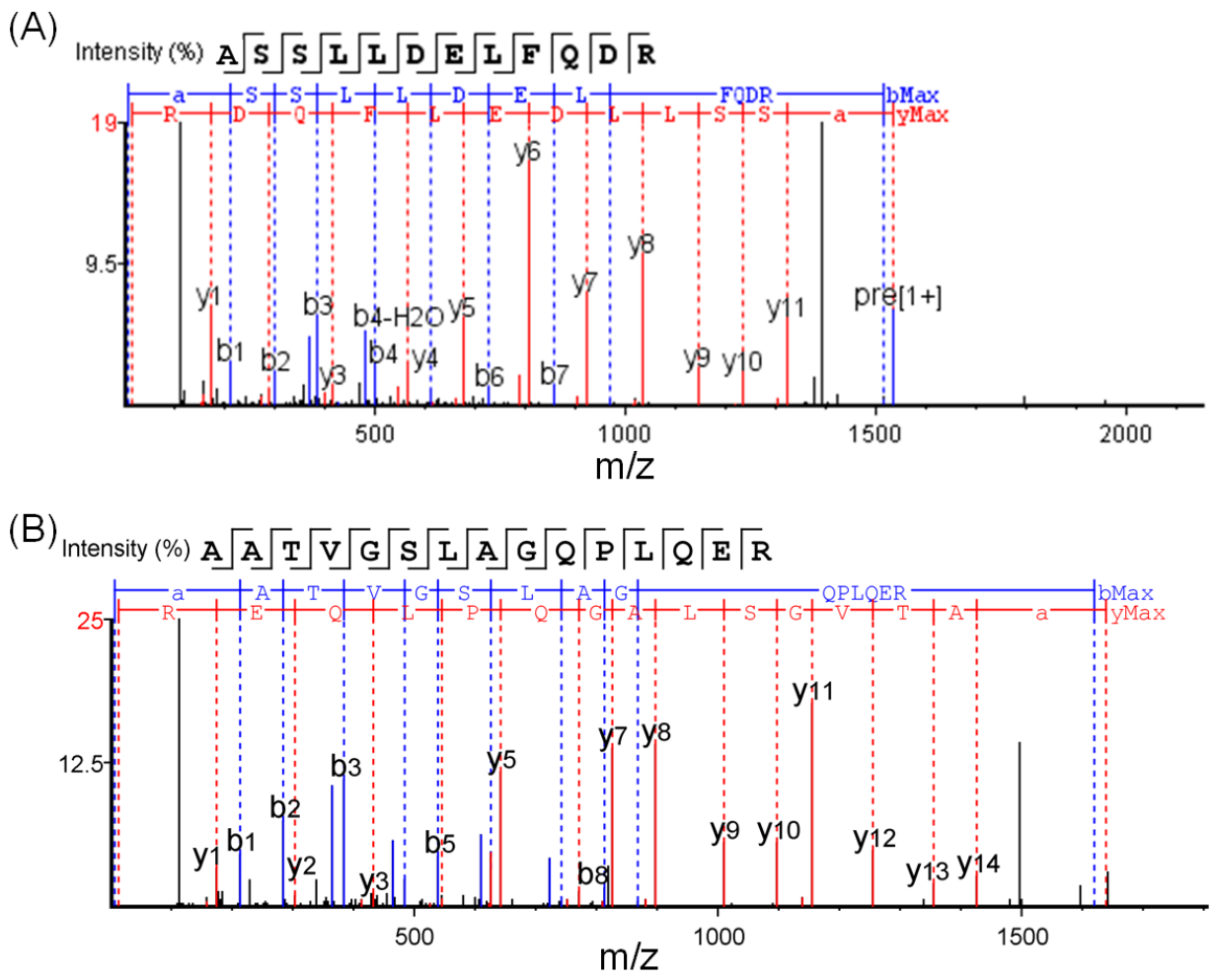

Figure 3. Example of tandem mass spectra for iDiLeu d0-labeled CLU peptide (A) and APOE peptide (B) in serum.

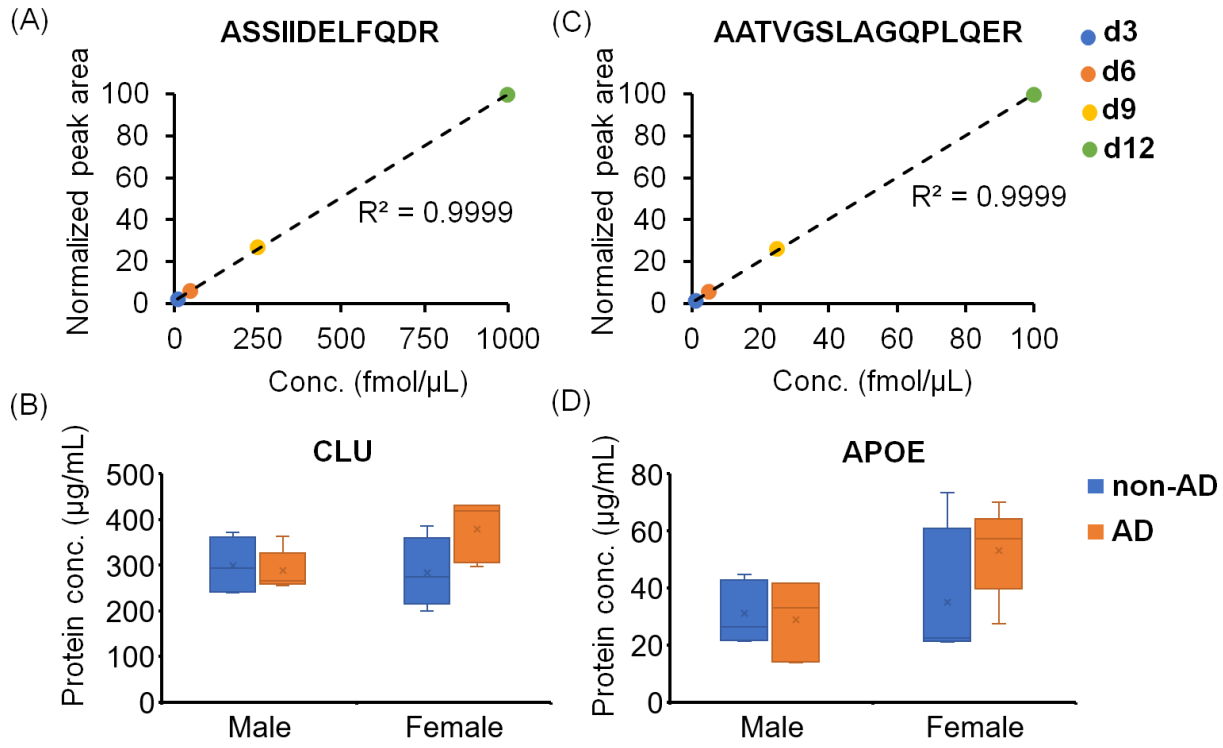

Figure 4. Calibration curves and the determination of absolute amounts of proteins CLU (A, B) and APOE (C, D) in serum samples of male and female in AD and non-AD participants. The boxes represent the 25% quartile, median and 75% quartile and the whiskers show the mean +/− standard deviation (B, D).