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Research Article

A multiplex assay of leptin, resistin, and adiponectin by immunoaffinity enrichment and targeted mass spectrometry

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ABSTRACT

Background: Leptin, resistin, and adiponectin are critical adipokines involved in the pathophysiology of obesity and its related disorders, including type 2 diabetes. Although these biomarkers have historically been quantified using immunoassays, the specificity of antibody-based methods has frequently been questioned. As a result, there is an increasing interest in developing reliable, multiplexed clinical assays that utilize mass spectrometry for improved accuracy. In this study, we present a multiplexed immunoaffinity liquid chromatography-tandem mass spectrometry (multi-IA-LC-MS/MS) assay designed for the sensitive and selective measurement of leptin, resistin, and adiponectin in human plasma.

Methods: Leptin, resistin, and adiponectin were selectively enriched from plasma samples using an antibody cocktail composed of monoclonal antibodies targeting each respective adipokine. The enriched adipokines underwent enzymatic digestion, and the resulting tryptic peptides were quantified using LC-MS/MS. The validated assay was subsequently applied to plasma samples collected from a cohort of subjects representing various weight categories, including normal weight, overweight, and obesity.

Results: The lower limits of quantification for the assay were determined to be 0.5 ng/mL for both leptin and resistin, and 50 ng/mL for adiponectin. Intra- day, inter- day, and total imprecision measurements were all < 15 %, while spike recovery consistently exceeded 83 %. Comparative analysis with individual immunoassays demonstrated strong correlation, with all correlation coefficients (r) being equal to or greater than 0.869. Notably, when comparing subjects with obesity to those with normal weight, there was an approximately nine-fold increase in circulating leptin levels and a ~1.6-fold decrease in circulating adiponectin levels.

Conclusions: A multi-IA-LC-MS/MS assay was developed for the simultaneous and sensitive measurement of leptin, resistin, and adiponectin in clinical samples. This quantitative method shows significant potential for applications related to obesity and could facilitate improved clinical management and understanding of obesity-related conditions.

Introduction

Adipokines are a significant class of signaling molecules, also referred to as cytokines, that are secreted by adipose tissue. They play a crucial role in mediating inter-organ communication and regulating various physiological processes, including appetite, energy metabolism,

Abbreviations: AAA, amino acid analysis; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; mAb, monoclonal antibody; MRM, multiple reaction monitoring; MS, mass spectrometry; multi-IA-LC-MS/MS, multiplexed immunoaffinity LC-MS/MS; QC, quality control; SOP, standardized operating protocol; XIC, extracted ion chromatogram.

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insulin sensitivity, and inflammation [1,2]. Obesity is recognized as a complex metabolic syndrome, where dysregulation of adipose function results in altered secretion of adipokines. This dysregulation contributes to chronic low-grade inflammation and plays a significant role in the pathogenesis of obesity-associated chronic diseases, including type 2 diabetes and cardiovascular diseases [1,3,4]. Among the adipokines, leptin, resistin, and adiponectin are the three most extensively studied signaling molecules, recognized as important regulators of both insulin sensitivity and inflammation [5]. Leptin significantly contributes to the regulation of metabolism, energy homeostasis, as well as neuroendocrine and immune function [6]. Resistin induces insulin resistance and impairs glucose homeostasis in rodents [7]; however, human resistin plays a role in inflammation, stress biology, and acts as a potential biomarker to assess obesity-associated disease state [8]. In contrast, adiponectin is an insulin-sensitizing adipokine that exhibits anti-inflammatory properties. The down-regulation of adiponectin has been identified as a mechanism through which obesity may contribute to insulin resistance and the development of type 2 diabetes [9]. Hence, leptin, resistin, adiponectin, and other adipokines collectively regulate inflammation, insulin action, and glucose metabolism both locally and systemically, establishing a molecular link between obesity and diabetes. A similar interplay of adipokines is also observed in the pathogenesis of cardiovascular diseases [10].

Given the complex interplay among adipokines, reliable and accurate multiplex assays for these molecules will be critical for enabling precise monitoring of the pathophysiology of obesity in clinical research. Several recent examples have highlighted the importance of monitoring multiple adipokines in the context of obesity. For instance, the relative levels of adiponectin compared to leptin have been proposed as indicators of adipose tissue dysfunction, which closely correlates with insulin resistance [11,12]. Similarly, an adiponectin-resistin (AR) index has been proposed as an indicator of metabolic risk in obesity, demonstrating a positive correlation with the risk of developing type 2 diabetes and the associated metabolic syndrome [13,14]. There is an increasing demand for multiplex methods that can simultaneously and reliably quantify the three adipokines, as these methods will enhance our understanding of their roles in obesity.

Current assays for leptin, resistin, and adiponectin are predominantly based on immunoassays, where the specificity of affinity reagents is often difficult to characterize and/or poorly characterized [15]. Moreover, most of the immunoassays have to be performed individually for each target, which is suboptimal for monitoring a panel of targets in large cohort studies. Although a multiplexed magnetic immunoassay has been reported [16], considerable deviations in the levels of the three biomarkers were observed from individual commercially-used ELISA methods, even with the same capture and detection antibody. Mass spectrometry (MS)-based assays have been increasingly recognized as promising alternatives for developing robust and standardized assays due to their unique advantage in detection specificity [15,17]. However, previous MS-based assays for leptin, resistin, and adiponectin have often fallen short in terms of analytical sensitivity and/or multiplexing capabilities [18–20]. For example, a recently described multiplexed LC-MS method showed a lower limit of quantification (LLOQ) of 111.85 ng/mL for measurement of leptin in serum [18], which is well above the endogenous levels in the majority of the serum samples from children analyzed in the study. Moreover, Tubbs *et al.* integrated immunoaffinity capture and MALDI-TOF MS to quantify only resistin with a linear range of 2.5–80 ng/mL [20], which may fail to capture the wide and diverse endogenous range among individual human samples.

Herein, we describe the development and validation of a multiplexed immunoaffinity liquid chromatography-tandem mass spectrometry (multi-IA-LC-MS/MS) assay designed for the simultaneous measurement of leptin, resistin, and adiponectin in plasma. This assay serves as a valuable tool for obesity-related clinical applications. We demonstrated the utility of this multiplex assay using human plasma samples from a clinical cohort comprising individuals with normal weight, overweight, and obesity, along with a subset of participants who underwent a dietary intervention for six months.

Materials and methods

Reagents and standards

Recombinant human leptin and adiponectin (Sino Biological, Wayne, PA), and recombinant resistin (R&D Systems, Minneapolis, MN) were utilized. Concentrations of protein stock solutions were determined by amino acid analysis (AAA) in two facilities with two batches (Molecular Structure Facility at UC Davis & AAA Service Laboratory, Inc., Davis, CA). Stable isotope labeled (SIL) peptides (Biosynth, Staad, Switzerland) were used as internal standards (IS), which was prepared by diluting to a final concentration of 0.1 μM in 30 % acetonitrile. For immunoaffinity enrichment, monoclonal antibodies (mAb) for all three antigens (R&D Systems, Minneapolis, MN) were biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific, Waltham, MA) to a final concentration of 0.1 mg/mL in PBS. Dynabeads™ MyOne™ Streptavidin T1 (Invitrogen, Waltham, MA) magnetic beads were used to capture biotinylated antibodies.

Calibration and quality control (QC) samples

For multi-IA-LC-MS/MS analysis, a 6-point calibration curve was prepared for each run, ranging from 0.5 to 100 ng/mL for leptin, 0.5 to 100 ng/mL for resistin, and 0.05 to 10 μg/mL for adiponectin, with serial dilution of the top point of the calibration curve in surrogate matrix, guinea pig plasma. A zero-point containing only internal standard (IS) was also injected. Three levels of QC samples were prepared by spiking different volumes of the stock solution into guinea pig plasma to a final concentration of 1 ng/mL for leptin and resistin, 0.1 μg/mL for adiponectin (low); 8 ng/mL for leptin and resistin, 0.8 μg/mL for adiponectin (medium); 80 ng/mL for leptin and resistin, 8 μg/mL for adiponectin (high). For each batch, the calibration regression coefficient of determination (i.e., r^2) was required to be > 0.98. The measured signals for the LLOQ calibrator should be within 20 % of the regression line, and all other calibrators should be within 15 % of the regression line. The observed concentrations of three levels of QC samples need to be within 20 % of their respective nominal concentrations.

Immunoaffinity capture, magnetic bead processing, and protein digestion

The concept for the multi-IA-LC-MS/MS assay is illustrated in Fig. 1A. Due to the vastly higher endogenous level of adiponectin compared to leptin and resistin, we employed a dual-capture format to capture leptin and resistin in one well, and adiponectin in another well in parallel with reduced plasma volume and reduced usage of antibody and beads. The three analytes were finally eluted into the same well. The detailed procedure is depicted in Supplemental Fig. 1. Briefly, 100 μL plasma aliquots for leptin and resistin measurements were added to the left half of a 96-deep-well plate, while corresponding10 μL plasma aliquots for adiponectin measurements were added to the right half of the plate. The sample IDs were arranged in a centrally symmetric pattern, with the layout on the right half being a central symmetry projection of the left half (Supplemental Fig. 1A). Each well was then diluted with PBS to a final volume of 400 μL. Two micrograms of equally mixed biotinylated anti-leptin and anti-resistin monoclonal antibodies (mAbs) (20 μL of 0.1 mg/mL) were added into wells containing 100 μL plasma, while 1 μg of biotinylated anti-adiponectin mAb (10 μL of 0.1 mg/mL) was added into wells containing 10 μL plasma. The sample plate was incubated at room temperature for 2 h at 550 rpm in a thermo mixer and transferred to a KingFisher Flex system (Thermo Fisher Scientific) for bead capture, wash and elution. Briefly, streptavidin magnetic beads were added to each well (10 μL beads/μg antibody), followed by incubation at room temperature for 1 h for bead capture with the mix speed set at low. After incubation, the beads were transferred to wash plates, and underwent two washes with 400 μL of PBS with 0.05 % Tween-20, followed by a final wash with 400 μL of PBS with the mix speed set at low. After the wash steps, beads were transferred to an elution plate. A

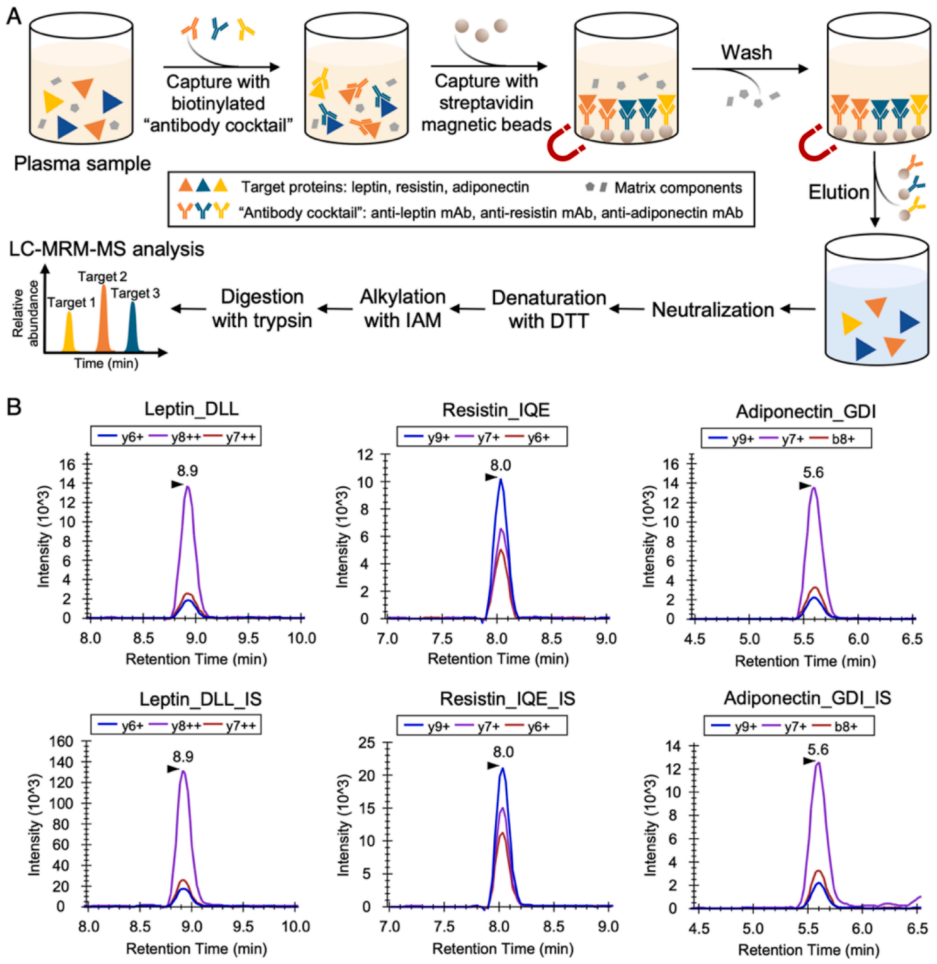

Fig. 1. The multi-IA-LC-MS/MS assay. (A) Schematic of multi-IA-LC-MS/MS assay workflow; **(B)** Representative extracted ion chromatograms (XICs) for endogenous leptin (7.6 ng/mL), resistin (11.6 ng/mL) and adiponectin (1.0 μg/mL) along with the corresponding internal standard (IS) peaks for each analyte from a typical human plasma sample.

two-step sequential elution was conducted to elute the three analytes from the same plasma samples into the same wells (Supplemental Fig. 1B). Briefly, 150 μL of eluent (25 mM HCl pH = 2.0) was added to the left half of the elution plate, while the remaining wells contained wash buffer (PBS). During the first elution step, the beads carrying leptin and resistin antibodies were eluted into the eluent, while the beads for adiponectin remained in the wash buffer. In the second elution step, the plate was rotated 180°, allowing the adiponectin from the same sample to be eluted into the same wells used in the first step. The mix speed was set at medium for elution. The pH of the eluate was raised immediately with 20 μL of 1 M Tris-HCl pH = 8, followed by adding 2 μL of 0.1 μM IS mix. Samples were reduced with 10 μL of 50 mM dithiothreitol (final dithiothreitol concentration of 2.75 mM) at 56℃ for 30 min, and were alkylated with 10 μL of 100 mM of iodoacetamide (final iodoacetamide concentration of 5.21 mM) at 37℃ for 30 min in the dark. Samples were digested at 37℃ for 6 h with 1 μg of trypsin. The digested samples were acidified with 1 % formic acid, and centrifuged at 18,000 × g under 4℃ for 30 min before LC-MS/MS analysis.

LC-MS/MS

All samples were analyzed using a trapping micro-LC-MS/MS system with an UltiMate 3000 LC system coupled to a Thermo Fisher TSQ Quantiva triple-quadrupole mass spectrometer as described previously [21]. A 50 μL injection volume of each sample was loaded onto a C8 column (15 × 2.1 mm, 3.5 μm, 100 Å) at flow rate of 1 mL/min for 0.5 min at 96 % mobile phase A (water: acetonitrile: formic acid of 98:2:0.1, v/v/v) and 4 % mobile phase B (water: acetonitrile: formic acid of 15:85:0.1, v/v/v) using the high-flow binary pump as sample trapping. Then the peptides are reversely eluted from trapping column to analytical C18 column (150 × 0.5 mm, 2.2 μm, 130 Å) at a flow rate of 25 μL/min, with a linear gradient from 5 % to 50 % mobile phase B over 10.5 min followed by a 0.1- min gradient from 50 % to 95 % mobile phase B. The gradient was held at 95 % mobile phase B for 1.5 min followed by a column equilibration step at 5 % mobile phase B for 1.5 min. A ZDV 6-port valve placed in the heated column compartment was utilized to coordinate operation of the two flow systems. The separation temperature was controlled at 40 °C. The spray voltage was 3500 V, the sheath gas was 8.0 Arb, the aux gas was 6.0 Arb. The capillary temperature was maintained at 325 °C, and vaporizer temperature was 50 °C. Multiple reaction monitoring transitions and collision energies were optimized for each analyte. Peak area ratios between each analyte and IS were used to quantify against a 6-point calibration curve with Skyline v.21.2 software.

Assay development and optimization

Assay development and optimization consists of (i) selecting optimal capture antibodies and protein standards; (ii) determining antibody amount and digestion time; and (iii) evaluating quantitative performance of the multi-IA-LC-MS/MS vs. the single format.

Optimal capture antibody was selected by evaluating capture performance of different sources of antibodies in both recombinant protein standards spiked into guinea pig plasma (i.*e.*, the surrogate blank matrix) at 40 ng/mL of leptin and resistin, and 4 μg/mL adiponectin, and representative human plasma samples containing low & high levels of

endogenous targets. Antibody amount of the selected antibody was assessed with 0.05 μg, 0.1 μg, 0.5 μg, 1 μg and 2 μg in the upper limit of quantitation (ULOQ) samples. Digestion time was evaluated under 2 h, 6 h, 8 h, 12 h, 18 h, and 24 h using two different human plasma pools. Optimal protein standards were selected by comparing the relative abundance of signature peptides in digests of recombinant proteins (5 μg) from two different vendors. The concentration of selected protein standard was accurately calibrated using quantitative AAA. The quantitative performance of the multi-IA-LC-MS/MS was compared to the single format (i.e., applying the same workflow with single antibody to capture each target individually in three separate assays) in three different human plasma pools.

Assessment of assay performance

Assessment of assay performance includes determination and evaluation of assay imprecision, dilution linearity, LLOQ, spike recovery, effects of clinically relevant interferents (i.e., triglyceride-rich lipoprotein, bilirubin), and pre- and post-preparation stability.

Assay imprecision was assessed using a 5x5 study, where five replicates of a human plasma pool were analyzed on each of five days. Dilution linearity was assessed by performing 2-fold serial dilutions of a spiked-in human plasma pool with high concentration of analytes (118.1 ng/mL of leptin, 130.3 ng/mL of resistin, and 11.3 μg/mL of adiponectin) with surrogate guinea pig plasma. The LLOQ was determined by analyzing plasma samples containing decreasing concentrations of the three analytes. The LLOQ was assigned as the lowest concentration that passed criteria (accuracy within ± 20 %, and inter-assay CV < 20 %). Spike recovery was evaluated by analyzing four different pools of human plasma samples before and after spiking with protein standards at 10 and 40 ng/mL for leptin, 10 and 40 ng/mL for resistin, 1 and 4 μg/mL for adiponectin. The effects of clinically relevant potential interferents were evaluated by spiking high concentration of interferent standards: triglyceride-rich lipoprotein, conjugated bilirubin, unconjugated bilirubin (Sun Diagnostics, New Gloucester, ME), or corresponding control buffer into four different pools of human plasma. Measurement of the three analytes in each sample was compared between high interferent group and control group. Pre-preparation stability of the three analytes in plasma samples was tested for 4 h at room temperature, 24 h at 4℃, or one to two freeze–thaw cycles; the post-preparation stability was tested under 4℃ for up to 2 days in autosampler, -20℃ for up to 4 weeks in freezer, or one to two freeze–thaw cycles.

Inter-laboratory validation was conducted between two MS laboratories at the University at Buffalo (UB) and Pacific Northwest National Laboratory (PNNL), which applied the same assay protocol to measure the three adipokines using the same set of 100 human EDTA plasma samples (BioIVT, Westbury, NY). For comparison to immunoassay, leptin, resistin, and adiponectin concentrations were assayed in a set of 100 human EDTA plasma samples by multi-IA-LC-MS/MS as well as three individual immunoassays [Human Leptin Quantikine ELISA kit (DLP00), Human Resistin Quantikine ELISA kit (DRSN00), Human Total Adiponectin Quantikine ELISA kit (DRP300); all from R&D Systems, Minneapolis, MN], following the manufacturer's instructions.

Clinical samples from subjects with normal weight, overweight, and obesity

De-identified leftover plasma samples were obtained from AdventHealth (Orlando, FL). The samples included a clinical plasma cohort from subjects with a wide range of body mass index (BMI), and longitudinal samples from a subset of subjects with obesity following a low-calorie diet intervention for six months (trial identifier: NCT00712127). The analysis of the de-identified samples in this study was qualified as non-human subjects research by the Institutional Review Board (IRB) at University at Buffalo.

Statistical analysis

Statistical analysis was performed using R (4.1.3) in RStudio (2023.03.0). Cross-sectional evaluation of protein concentrations across normal weight, overweight, and obese cohorts was performed using a non-parametric Kruskal-Wallis ANOVA following analysis of pairwise heteroscedasticity and normality using the F test and the Shapiro-Wilk test, respectively. Post-hoc analysis was performed by Dunn's test. Longitudinal evaluation of protein concentration before and after six months low-calorie diet for the cohort with obesity was performed using the Wilcoxon signed-rank test. Alternative hypothesis for the Wilcoxon signed-rank test was that the true location shift is not equal to zero. P values were adjusted for multiple comparisons using the Holm method as necessary and evaluated at a significance level of 0.05.

Results

Multi-IA-LC-MS/MS assay development

The development of a targeted multi-IA-LC-MS/MS method for the three adipokines was carried out similarly as described previously [22]. Briefly, initially 4 to 5 candidate signature peptides for each protein were selected based on various sequence features (e. g., uniqueness, likelihood of PTMs, and LC-MS detectability). The targeted MS conditions for each candidate peptide were determined using an on-the-fly orthogonal array optimization (OAO) method [22]. The candidates with optimized targeted MS conditions were then evaluated in human plasma samples containing endogenous targets in terms of sensitivity and stability. In the end, two signature peptides, one for quantification and one for confirmation, were selected per protein. The relative area ratio of the quantification peptide vs. the confirmation peptide was used to assess the presence of interference in each run. For each signature peptide, signals from both the heavy isotope-labeled synthetic peptide, as IS, and the endogenous peptide were monitored by LC-MS. Peptide DLLHVLAFSK for leptin, IQEVAGSLIFR for resistin and GDIGETGVPGAEGPR for adiponectin were chosen as quantitative signature peptides with three MRM transitions monitored simultaneously (Fig. 1B). MRM transitions and conditions are listed in Supplemental Table 1.

To select the capture antibodies for immunoaffinity enrichment, two monoclonal antibodies from different vendors for each target were evaluated. The antibodies were biotinylated and tested in both recombinant protein standards spiked into a surrogate blank matrix (guinea pig plasma) and representative human plasma samples exhibiting low or high levels of endogenous targets (Supplemental Fig. 2). After assessment, the following mAbs were selected due to their superior capture efficiency and reproducibility: anti-leptin (clone 44802), anti-resistin (clone 184305), and anti-adiponectin (clone 166126), all sourced from R&D Systems. With the final selection of mAbs confirmed, several key parameters of the immunoaffinity binding process, including antibody amount, antibody-to-bead ratio, wash conditions, elution techniques, and digestion conditions, were optimized to maximize the recovery of all three targets (refer to the Methods section for specific details). A digestion duration of six hours was determined to be optimal, yielding high peptide outputs with excellent reproducibility.

To enable accurate quantification, it is essential to use protein standards with precisely assigned concentrations for the preparation of calibrators. Although most manufacturers claim high purity for their recombinant proteins, discrepancies frequently occur between the manufacturer-provided protein content and the actual values. These discrepancies can arise due to various factors, including measurement errors or contamination from non-protein components [23]. Therefore, we conducted a quantitative amino acid analysis (AAA) involving triplicate, blinded analyses from two different facilities to calibrate the purity of the three recombinant protein standards. The measured contents from the AAA revealed that the actual protein concentrations were 70 %, 39 %, and 103 % of the manufacturer-labeled values for leptin, resistin, and adiponectin, respectively (Supplemental Table 2). As no impurity proteins were detected in any of these products, we speculate that the impurities primarily originated from buffer salts used during the purification process. The calibration curves, adjusted with the corrected concentrations of the standards, are shown in Supplemental Fig. 3.

To determine whether the quantitative performance of the multi-IA-

LC-MS/MS assay is equivalent to that of the single format, we conducted an equivalence study using three different human plasma pools. The results demonstrated that the multiplexed format achieved consistent quantification for all three targets within ± 12 % quantitative deviation and exhibited good precision, indicating that it is statistically equivalent to each of the three single formats (Supplemental Table 3). Furthermore, the multiplexed format significantly increased throughput compared to the single format. With a 15-minute LC-MS/MS cycle time, the multi-IA-LC-MS/MS assay can analyze up to 96 samples per day, while the sample preparation time for 96 samples is approximately 12.5 h.

Analytical validation

The imprecision of the assay was evaluated through a 5 × 5 study, analyzing five replicates of a representative human plasma pool each day over five different days. The intra- day, inter- day, and total imprecision CVs for the three targets were found to be below 11.2 %, 12.7 %, and 11.8 %, respectively (Supplemental Table 4). The lower limits of quantification (LLOQ) were determined to be 0.5 ng/mL for leptin, 0.5 ng/mL for resistin, and 50 ng/mL for adiponectin, all achieving accuracy within ± 20 %, with inter-assay CV lower than 11.8 % (Supplemental Table 5). Additionally, a dilution linearity experiment demonstrated that dilutions of human plasma samples up to 128-fold with guinea pig plasma resulted in acceptable quantitative accuracy, within 20 % of quantitative error compared to the undiluted sample (Supplemental Table 6). Spike recoveries ranged from 83.5 % to 98.2 % for leptin, 87.6 % to 100.3 % for resistin, and 88.2 % to 109.8 % for adiponectin, measured from four different human plasma pools (Supplemental Table 7). Interference testing showed no significant interferences with the quantification of leptin, resistin, and adiponectin, with interference levels remaining below 15 % in plasma samples spiked with triglyceride-rich lipoproteins up to 500 mg/dL or bilirubin (both conjugated and unconjugated) at levels up to 40 mg/dL (Supplemental Table 8). Stability assessments indicated that all analytes remained stable in EDTA plasma for at least 4 h at room temperature, 24 h at 4 ℃, or through one to two freeze–thaw cycles prior to sample preparation. Prepared samples were stable for at least two days at 4 ℃ in the autosampler, four weeks at −20 ℃ in the freezer, or through one to two freeze–thaw cycles (within 20 % bias from baseline/fresh) (Supplemental Table 9). No carryover was detected immediately after injecting a plasma sample spiked with 100 ng/mL leptin, 100 ng/mL resistin, and 10 μg/mL adiponectin (i.e., at the assay upper limit of quantification). Table 1 summarizes the main performance characteristics for the assay.

Inter-laboratory validation and comparison with immunoassays

An inter-laboratory validation study was conducted to measure the three adipokines in a cohort of 100 individual human plasma samples using the same assay standard operating protocol (SOP) between two MS laboratories at the University at Buffalo (UB) and Pacific Northwest National Laboratory (PNNL). The results showed a strong correlation, with the Pearson correlation coefficient (r) ranging from 0.866 to 0.965 (Fig. 2A). However, some deviations from the correlation line were noted at elevated target concentrations. While the linearity of the calibration curve and QCs were within acceptance criteria in both laboratories, these differences may stem from variations in sample preparation, particularly during the immunocapture pull-down step. Overall, the study demonstrated that a robust assay SOP can be effectively transferred between different MS labs, as evidenced by the high correlation between results from the two laboratories analyzing the same set of samples. This suggests significant potential for the broad application of the developed LC-MS assay.

To assess the correlation of results between the multi-IA-LC-MS/MS assay and individual immunoassays, a total of 100 individual human plasma samples were analyzed by both methods (Fig. 2B). The comparison revealed a strong correlation, with Pearson correlation coefficients ranging from 0.869 to 0.981. The observed differences in absolute concentrations determined by the two methods can likely be attributed to the use of different capture antibodies and the distinct quantification platforms employed (LC-MS versus ELISA). Overall, the developed multi-IA-LC-MS/MS assay demonstrated comparable quantitative results with the individual immunoassays, effectively enabling the simultaneous quantification of three adipokines from a single sample.

Measurement of leptin, resistin, and adiponectin in an obesity study cohort

With the development and validation of the multi-IA-LC-MS/MS assay, we proceeded to demonstrate its utility by quantifying the levels of leptin, resistin, and adiponectin in a clinical cohort consisting of plasma samples from 256 subjects with varying body mass index (BMI) values. The demographic characteristics of the participants are summarized in Table 2. Additionally, we conducted a longitudinal analysis of pre- and post-intervention plasma samples from a subset of 34 individuals in the obesity group (BMI $\geq$ 30 kg/m^2), who underwent a low-calorie diet intervention over a period of six months.

We initially compared the levels of the three adipokines in three subject groups: normal weight, overweight, and obesity. As illustrated in Fig. 3A and Supplemental Table 10, both leptin and adiponectin exhibited significant differences in concentrations across all three groups (all $p < 0.05$). In contrast, resistin levels did not show significant variation among the three groups. These findings suggest that elevated levels of leptin and decreased levels of adiponectin may be closely associated with BMI levels, while the relationship between resistin and obesity remains less clear. This aligns with previous literature, reinforcing the notion that leptin and adiponectin are more related to obesity-related conditions [24–27]. We further examined the assay specificity across the clinical samples by evaluating the relative abundance ratio of two transitions for each analyte. The transition ratios for calibration curves and QC samples, as well as for different groups of clinical samples, demonstrated good consistency (Supplemental Fig. 4). These results provide additional evidence of the high specificity of the developed final assay for each analyte.

Next, we investigated the effect of a weight loss intervention on the levels of circulating adipokines in a subset of 34 subjects with obesity who underwent a restricted low-calorie diet. The subjects were stratified into three groups based on the extent of weight loss: those with > 10 %, 5–10 %, and < 5 % weight loss. As illustrated in Fig. 3B, we observed significantly reduced levels of leptin and increased levels of adiponectin and resistin in the group that experienced more than 10 % weight loss. Although the increase in resistin was relatively small, this observation is consistent with previous reports regarding weight loss interventions [28]. For participants who experienced 5–10 % weight loss, leptin and adiponectin still exhibited significant differences following the intervention, albeit to a lesser extent. In contrast, among those with less than 5 % weight loss, none of the analyzed adipokines showed significant differences after the intervention. It is important to note that the relatively small number of subjects in each category may limit the statistical power of these findings, even if the analytes are responsive to the

Table 1
Performance characteristics of the multi-IA-LC-MS/MS assay.

Analyte	Intra-assay imprecision	Inter-assay imprecision	Total imprecision	LLOQ	Spike recovery
Leptin	5.0 %-9.7 %	5.8 %-12.1 %	10.8 %	0.5 ng/mL	83.5 %-98.2 %
Resistin	4.4 %-9.1 %	2.4 %-9.1 %	8.6 %	0.5 ng/mL	87.6 %-100.3 %
Adiponectin	5.6 %-11.2 %	7.8 %-12.7 %	11.8 %	50 ng/mL	88.2 %-109.8 %

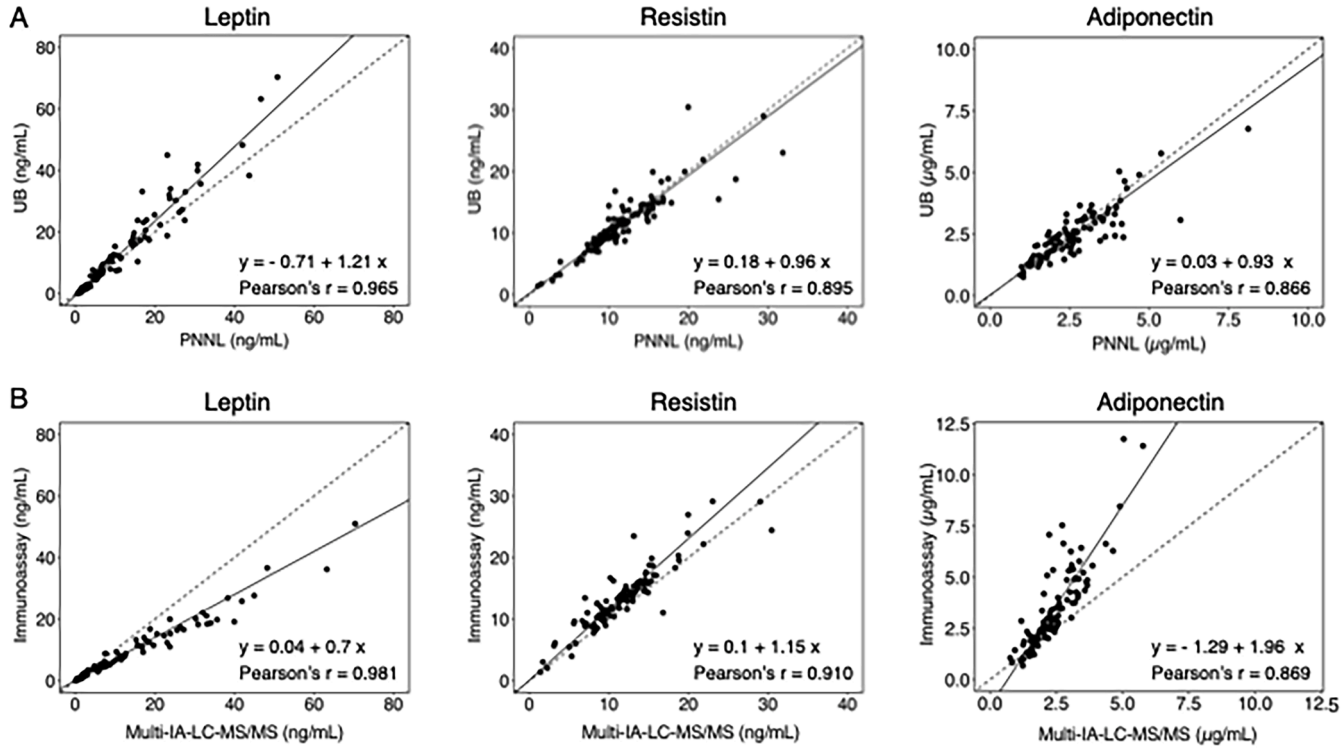

Fig. 2. Inter-lab validation of the multi-IA-LC-MS/MS method and comparison of multi-IA-LC-MS/MS with immunoassays by parallel measurement of leptin, resistin, and adiponectin in human plasma samples (n = 100). (A) Inter-laboratory validation of the multi-IA-LC-MS/MS method between two mass spectrometry laboratories at UB and PNNL; **(B)** Multi-IA-LC-MS/MS method compared with three individual immunoassays. All data were fit using Deming regression. The solid line indicates the regression line.

Table 2
The demographic characteristics of the participants.

	Normal weight (n=36)	Overweight (n=62)	Obesity (n=158)
BMI range, (kg/m^2)	18.5–24.9	25–29.9	≥30
BMI (kg/m^2), mean (SD)	22.6 (1.6)	27.5 (1.5)	40.7 (6.9)
Male, n (%)	7 (19.4)	20 (32.3)	25 (15.8)
Age (yr), mean (SD)	44.3 (6.5)	45.6 (6.5)	46.1 (6.4)

intervention. Overall, the data indicate that both leptin and adiponectin are robust markers of obesity, demonstrating substantial responses to weight loss. These findings further highlight the utility of the developed multiplex assay within clinical cohorts.

Discussion

We present the development and validation of a multiplexed IA-LC-MS/MS assay for the simultaneous measurement of three adipokines—leptin, resistin, and adiponectin—in human plasma. The assay involves the immunoaffinity enrichment of these adipokines, followed by tryptic digestion and quantification of the signature peptides for each target using LC-MS/MS. The LLOQs were determined to be 0.5 ng/mL for both leptin and resistin, and 50 ng/mL for adiponectin in plasma. This represents a significant improvement in analytical sensitivity compared to previously published LC-MS methods for these three adipokines [18–20]. Comparing the multi-IA-LC-MS/MS assay with standard LC-MS/MS without antibody capture, we observed estimated improvements in LLOQs by 50- to 100-fold for the three target adipokines. The LLOQs achieved in our assay are significantly lower than the reported endogenous interval ranges for these adipokines in human plasma, indicating that the assay is sufficiently sensitive to reliably quantify endogenous levels. The validated method demonstrates acceptable performance regarding intra- and inter-assay imprecision, dilution linearity, spike recovery, interference effects, and stability. Additionally, this assay supports the analysis of up to 96 samples per day, with a preparation time of 12.5 h. The method has shown to be transferable and easily standardized across laboratories. As evidenced by an inter-laboratory validation study, two different MS laboratories applying the same SOP achieved highly correlated measurements of the three adipokines in the same set of human plasma samples. The developed assay was employed to measure circulating levels of leptin, resistin, and adiponectin in 256 BMI-matched human plasma samples, revealing significantly altered levels of leptin and adiponectin among subjects with normal weight, overweight, and obesity.

Furthermore, the quantitative values of the three adipokines (leptin, resistin and adiponectin) measured by the multi-IA-LC-MS/MS assay showed strong correlation with those obtained from three individual immunoassays. LC-MS-based assays provide several advantages over traditional immunoassays, including enhanced selectivity and specificity, as well as an excellent capability for multiplexing. As a result, the requirements for the specificity of capture antibody reagents are less stringent compared to ligand-binding assays (LBAs), making it unnecessary to rely on high specificity from antibody reagents. The development of the tri-plex assay was both rapid and straightforward. Moreover, monitoring multiple transitions from the same peptide across calibration/QC samples and clinical samples ensures specificity for the target measurements. Consequently, the multi-IA-LC-MS/MS assay emerges as a valuable complementary tool for quantifying biomarkers in clinical cohorts, as well as for the validation and optimization of immunoassays. This advancement contributes critically to assay standardization, optimization, and harmonization, ultimately enhancing the rigor of biomedical research.

It is important to acknowledge several limitations of this study.

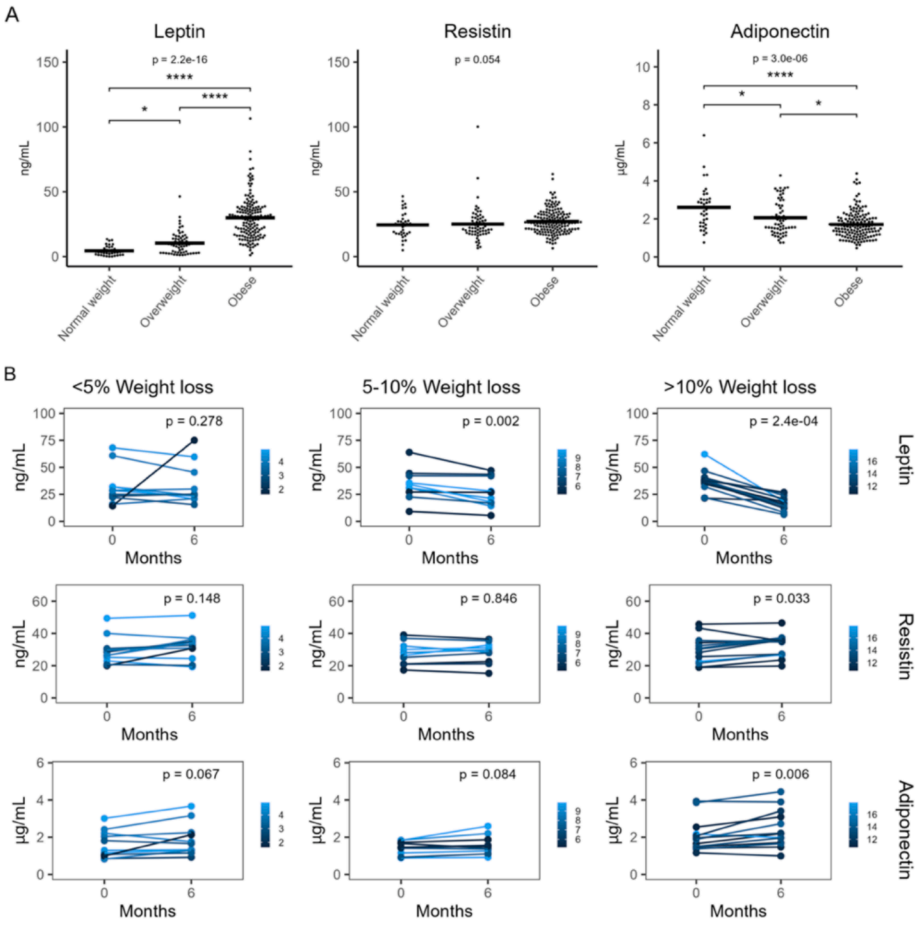

Fig. 3. Measurement of leptin, resistin, and adiponectin in a clinical plasma cohort with the multi-IA-LC-MS/MS method. (A) Distribution of concentrations of leptin, resistin, and adiponectin in subjects with normal weight (n = 36), overweight (n = 62), and obesity (n = 158). Multiple comparisons were performed using the Kruskal-Wallis test (* $p < 0.05$; **** $p < 0.0001$); **(B)** Changes in circulating levels of leptin, resistin, and adiponectin in 34 subjects with obesity following a 6-month low-calorie diet intervention. The subjects were further stratified into three groups: subjects who experienced > 10 % (n = 13), 5–10 % (n = 10), and < 5 % (n = 11) weight losses after 6 months. The figure legend represented % weight loss for each subject.

Firstly, we utilized commercially available capture antibodies, which may pose a potential risk due to the possibility of discontinuation. To address this concern, we are currently in the process of producing and validating in-house monoclonal antibodies via hybridoma technology. Upon successful equivalence tests, the selected hybridomas will be deposited into a hybridoma bank for broader availability. Secondly, batch-to-batch variability is a common challenge associated with any assays that utilize capture reagents. Therefore, it is crucial to meticulously evaluate and characterize different batches and lots of antibodies to ensure quantitative consistency. Additionally, instead of using SIL-full-length proteins as the internal standard, we opted for SIL peptides in this study. While this choice prevents accounting for bias and variability during immunoaffinity enrichment, we still achieved a commendable total assay imprecision (< 15 % CV). Considering the large size of the three protein analytes, employing SIL peptides as an internal standard can be more cost-effective, provided that the performance of immunoaffinity capture is rigorously examined.

Conclusion

This study demonstrated the feasibility of using immunoaffinity enrichment combined with tandem mass spectrometry to simultaneously quantify three critical adipokines: leptin, resistin, and adiponectin. We emphasized the significance of these adipokine levels in plasma concerning obesity and the effects of weight-loss interventions. The developed assay holds substantial potential for applications in clinical research related to obesity and offers a new benchmark method for quantifying adipokines in clinical samples alongside traditional immunoassays. The multiplexed format showcased in this study could facilitate the inclusion of additional adipokines and other obesity biomarker candidates in a single analysis, paving the way for future studies aimed at comprehensive metabolic profiling.

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Ethics statement

The analysis of the de-identified samples in this study was qualified as non-human subject research by the Institutional Review Board (IRB) at University at Buffalo.

CRediT authorship contribution statement

Jie Pu: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Xinxin Yang:** Writing – review & editing, Investigation. **Tai-Tu Lin:** Writing – review & editing, Validation, Investigation, Formal analysis. **Thomas L. Fillmore:** Writing – review & editing, Validation. **Marina A. Gritsenko:** Writing – review & editing, Validation. **Shane S. Kelly:** Writing – review & editing, Validation, Formal analysis. **Adam C. Swensen:** Writing – review & editing, Validation. **Tujin Shi:** Writing – review & editing, Validation, Conceptualization. **Stephen R. Master:** Writing – review & editing, Conceptualization. **James P. DeLany:** Writing – review & editing, Resources.

Bret H. Goodpaster: Writing – review & editing, Resources. **Wei-Jun Qian:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jun Qu:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2025.01.003.

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