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# Development of Recombinant-Based Mass Spectrometric Immunoassay with Application to Resistin Expression Profiling

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**This report addresses the need for additional assays for human resistin (hRES) by developing a rational progression of the mass spectrometric immunoassay to incorporate recombinant proteins. The recombinant-based hRES mass spectrometric immunoassay (RES-MSIA) was initially developed for the qualitative analysis of the human resistin homodimer from normal (healthy) plasma samples. The method involved selective extraction and detection of both endogenous and recombinant resistant proteins. RES-MSIA was then applied to the rigorous quantification of resistin. The resistin standard addition curve was constructed from serially diluted concentrations of rhRES using endogenous hRES, inherent in the human plasma, as the internal reference standard (IRS). The roles of endogenous and recombinant resistin were subsequently reversed, using rhRES as the IRS during RES-MSIA quantification. Concurrently, the relative ratio of hRES to rhRES was used as an ancillary technique to rapidly determine the relative concentration of hRES in each of plasma samples. Overall, normal hRES levels determined by RES-MSIA were found to be comparable to those selected and determined by ELISA. With regard to gender, female donor samples were slightly elevated over males. Four single cardiac samples were analyzed and found to have hRES concentrations approximately three times that of the normal. The recombinant-based RES-MSIA is rapid and is amendable to parallel high-throughput robotic processing of resistin related disease cohorts.**

The progression of proteomics into clinical protein analyses is predicated in approaches that not only enable biomarker discovery but also transcend the original qualitative data to relative/absolute quantitative assemblies, in essence, providing the ability to attain detailed informatics on the biomarker or protein target of interest. Toward this clinical proteomics goal, the mass spectrometric immunoassay (MSIA) approach integrates affinity capture in conjunction with mass spectrometry to detect and identify target analytes via the inherent mass of singular or multiple biomolecules.[1] Shown in Figure 1 is an overview of the MSIA approach. Here, MSIA-tips, antibody-derivatized affinity microcolumns stationed at the orifice entrance of wide-bore pipet tips, are utilized to capture proteins from biological solutions

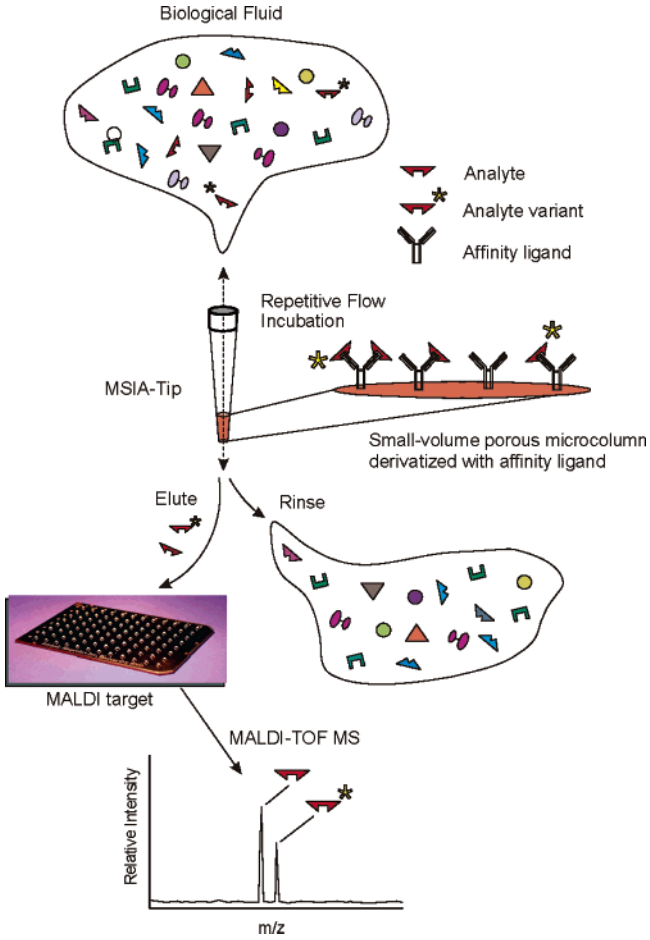


**Figure 1.** Diagram of MSIA process. A biological sample is repetitively passed though the MSIA-Tip, performing immunoaffinity capture of the targeted analytes. While nonspecific components within the biological fluid are rinsed away, the concentrated target analyte is eluted onto a MALDI target for mass spectrometric analysis.

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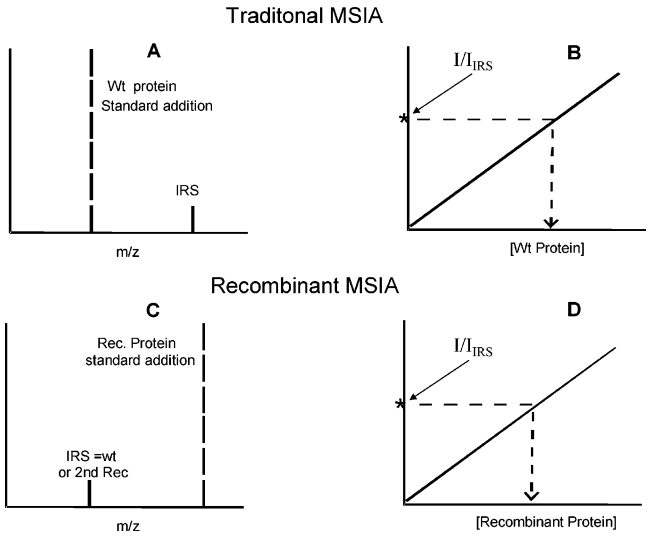


**Figure 2.** General MSIA compared. (A) Traditional MSIA: a progressive addition of wt protein standards is normalized to IRS (homologue or analogue of wt protein, shown at left side of drawing) and used to generate a working curve, versus (B) recombinant-based quantitative MSIA where the working curve is generated from standard addition of recombinant protein integral normalized to endogenous wt or a secondary recombinant protein.

consisting of microliter to milliliter volumes. The selectively extracted target protein(s) are then eluted, using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) matrix to dissociate the affinity interaction, onto a target array for subsequent mass spectrometric analysis. The methodology has been used to detect endogenous proteins to uncover structural features resulting from protein modifications.[3,7−12] The MSIA approach is dynamic, combining robotic liquid sample handling with a high-throughput analytical system analysis for functional protein screening and biomarker population studies.[2,4−6,8,12,13] The MSIA approach can also be used to quantify target proteins from biological media. Shown in Figure 2A is the schematic of wild-type target protein at a known molecular weight, with its peak intensity normalized to a protein variant (internal reference standard, IRS) shifted in mass from the wild-type signal. This ability to simultaneously resolve mass species permits determination of target proteins at either absolute quantitative values, using a working curve (Figure 2B), or relative quantification using multiple analyte analysis.[1−3,10,11] In the conventional MSIA approach, these internal reference standards are modified proteins, i.e., natural homologues (from another animal species of close homology) or chemical variant analogues, of the target wild-type (wt) protein that retain the immunoaffinity interaction. The inevitable unavailability of variant wt proteins as designated internal reference standards for low-level protein analyses necessitates a modified MSIA approach toward use of recombinant proteins as secondary binding partners. The advantages of recombinant proteins for MSIA are not only availability but also in their expression. Generally produced and purified at standard metrics from *Escherichia coli*, recombinant proteins provide a unified sequence free of multiple forms that have a N-terminal methionine (or other short leader sequence) to differentiate their mass from that of the wt protein, all traits of great importance for the MSIA process. Recombinant-based MSIA makes ready use of the immunoaffinity interaction originating between recombinant elicited antibody and recombinant protein. As shown in Figure 2C, the recombinant-based MSIA diagram uses increasing concentrations of recombinant protein normalized to wt protein, present in the nascent biological fluid, to generate integral peak values ($I_{rhRES}/I_{wt}$). The intrinsic ability of the MSIA process is used to mass resolve these species simultaneously, allowing quantitative measurement of a targeted endogenous protein from a working curve (Figure 2D). As a model application, resistin (RES) embodies interesting features from which to explore and develop a recombinant-based MSIA analysis.

Plasma resistin (RES or RETN) levels (~5 ng/mL) in healthy humans have recently been positively correlated with inflammatory markers and coronary artery calcification.[14] Using quantitative enzyme immunoassay, independent of C-reactive protein, RES was deemed predictive of atherosclerotic cardiovascular disease (CVD).[14] Also in the same study, plasma resistin levels were observed to be modestly, but significantly, higher in women than men. The origin of human resistin has been debated. While some studies suggest resistin is expressed by adipose tissues, other investigations have shown that human resistin is expressed from blood mononuclear cells,[15−17] indicating the link or role in inflammation. In vitro treatment of human peripheral blood mononuclear cells with proinflammatory cytokines, i.e., interleukin-1 (IL-1), IL-6, tumor necrosis factor α, or lipopolysaccharides, has been shown to enhance resistin mRNA expression.[18] Other in vitro studies have shown that resistin activates endothelial cells with concomitant production of adhesion molecules, e.g., endothelin-1, and chemokines.[19,20]

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Resistin has also been studied in humans as a potential link between obesity and the development of insulin resistance in type 2 diabetes. While several investigations demonstrate significant correlation,[21−26] other studies report no correlation between resistin and insulin resistance in type 2 diabetes.[26−31] These conflicting findings demonstrate the need for further investigations and development of additional assays with greater information content to better understand the complex biology of resistin in the human pathophysiology of obesity and diabetes.[32−34]

The adipokine resistin was discovered using genomic approaches by three separate groups.[35−37] Resistin is a member of a family of cysteine-rich secretory proteins, the resistin-like molecules (RELMs) whose other family members include RELM-α/FIZZ1 and RELM-$\gamma$, and RELM-$\beta$/FIZZ2.[37−39] The amino acid sequence of resistin contains 108 residues with a potential N-terminal signal sequence (residues 1−18) and a motif containing 11 cysteine residues, 10 of which are characteristic of the RELM family,[35−37] thereby providing a processed protein (consisting of residues 19−108) with an average mass of 9,553.9 Da. Resistin is proposed to be secreted as a disulfide bond linked homodimer with an average mass of 19 104.8 Dal and potentially exists in higher order aggregate structures.[40−43] Recombinant human resistin (rhRES) is obtained from *E. coli* as a purified 19 454-Da dimeric protein that consists of two 92-amino acid polypeptide chains joined by a single disulfide bond.[40,43] These mass differences easily distinguish rhRES from wt RES for mass spectrometric analysis, providing an essential characteristic from which to construct a recombinant-based RES-MSIA.

Nascent intact human resistin has not been detected in human serum or plasma using MALDI-TOF mass spectrometry nor has resistin been surveyed across other CVDs or other inflammatory disease cohorts as a putative candidate biomarker. With the clear need to address additional assays for resistin, this paper develops a recombinant hRES-based mass spectrometric immunoassay (RES-MSIA) for the homodimer of human resistin from normal plasma samples with subsequent resistin quantification using recombinant human resistin as an internal reference standard. The resistin assay was later used to challenge a plasma cohort (normal and cardiac samples) in a moderate throughput evaluation and quantification study. The plasma cohort was selectively stratified for RES levels, while the cardiac donors were prequantified for cardiac troponin I (cTnI), prior to RES-MSIA analysis.

## EXPERIMENTAL SECTION

**Material and Sample Preparations.** Affinity purified polyclonal anti-rhRES antibody (Catalog No. AF1359, R&D Systems, Minneapolis, MN) was conjugated to preactivated acylimidazole MSIA-Tips as previously described.[10,11] All individual human plasma samples were obtained from ProMedDX (Norton, MA) under an Institutional Review Board (IRB) approved protocol and Informed Consent. Heparin-treated plasma samples were stored at −78 °C prior to use, at which time they were thawed at room temperature, mixed, and centrifuged for 2 min (at 7000 rpm, 2500*g*) to remove particulates from the solution. rhRES was obtained purified from *E. coli* (Catalog No. R1135, Leinco Technologies, Inc., St. Louis, MO).

**Recombinant-Based RES-MSIA.** Four hundred microliters of plasma was mixed with 1.5 mL of HEPES-buffered saline physiological buffer containing EDTA and surfactant P20 (HBS-EP: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) and aliquoted into a 96-deep well microtiter plate (1.9 mL into each well). rhRES (100 $\mu$L) was added to the diluted samples, mixed, and used as the internal reference standard. RES extraction was performed with a multichannel electronic pipet by repetitive 250-$\mu$L aspiration and dispensing steps through antibody-derivatized MSIA-Tips mounted on the eight-channel head, in the following sequence: HBS-EP buffer (2 times), plasma samples (70 times), HBS-EP (5 times), $H_2O$ (5 times), 25% acetonitrile in 2 M ammonium acetate (2 times), and $H_2O$ (5 times). Following the final water rinse, the MSIA-Tips were blotted with a Kimwipe in preparation for the elution step. Retained proteins were eluted manually with a 20-$\mu$L pipet by deposition of the captured proteins from the MSIA-Tips onto a MALDI target using 6.5-$\mu$L aliquots of MALDI matrix (saturated aqueous solution of α-cyano-4-hydroxycinnamic acid, in 33% (v/v) acetonitrile, 0.4% (v/v) trifluoroacetic acid), aspirated into each affinity pipet tip, and, after a 10-s time delay, dispensed directly onto a 96-well formatted plate. The target eluents were air-dried allowing matrix crystallization. MS analysis was performed on a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) in linear delayed-extraction mode with a 2.25-kV drawout pulse (450 ns) with full accelerating potential of 19.5 kV. [The mass spectrometer was externally calibrated using singly and doubly charged species of bovine chymotrypsinogen ($m/z$ = 25 666).] Two hundred-fifty laser shots were summed and saved as five individual spectra per


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sample. Mass range 16−26 kDa was used for peak normalization/integration of the recombinant and endogenous resistin mass specific species, respectively.

**Enzyme-Linked Immunosorbant Assay (ELISA) Analysis.** Selected plasma samples were analyzed for hRES levels at R&D Systems using standard ELISA procedures (Catalog No. DRSN00, R&D Systems).

**Working Curve Generation.** For generation of the rhRES working curve, rhRES standards (100 $\mu$L in HBS-EP buffer) were used as additions to six 1.5-mL HBS-EP buffer aliquots. A 400-$\mu$L aliquot of normal human plasma was added to the mixture and used as the IRS. Six standard samples containing rhRES at concentrations of 2.5, 5, 10, 20, 40, and 80 ng/mL were prepared from a 1000 ng/mL stock and assayed in parallel using a multichannel pipet fitted with anti-rhRES MSIA-Tips as described in the RES-MSIA above. Five 200 laser-shot mass spectra were taken for each sample. Signals (at peak heights) for hRES and rhRES were integrated to baseline and the rhRES normalized to the hRES using IBI Proteome Analyzer (Intrinsic Bioprobes, Inc., Tempe, AZ) software. Normalized values from the eight samples were averaged and plotted against the rhRES concentration. The resulting response versus rhRES were correlated for linear least-squares fit using Sigma Plot (SPSS Inc.) with error bars reflecting the standard deviation of each point.

**Application to Human Plasma Samples.** ProMedDx samples (400 $\mu$L) from nine normal donors and four cTnI stratifed cardiac samples (AxSYM, Automated Microparticle Enzyme Immunoassay Instrument System, Abbott, Abbott Park, IL) were prepared as described in the previous section, with the exception that RES-MSIA analysis was performed at two rhRES concentrations, first at 8 ng/mL and then at 40 ng/mL, for a two-point quantitative analysis. Five spectra (200 laser shots each) were taken from various locations within the same sample target for each of the 13 samples (9 normal plus 4 cardiac). The generated data were treated essentially as described in the previous section with the exception that hRES was normalized to rhRES and a concentration factor (5-fold differential from the 8 ng/mL IRS of the working curve) used to multiply the 40 ng/mL rhRES normalized values. The nomalized values ($y$-values) for the two-point referenced spectra (five spectra for each of the four cardiac samples run in parallel with the IRS at 8 and 40 ng/mL) were averaged, adjusted for relative rhRES IRS concentration, the concentration of RES values obtained (calculated from least-squares equation), and the endogenous RES concentration values averaged.

## RESULTS AND DISCUSSION

**Recombinant-Based RES-MSIA.** Currently available quantitative human resistin solid-phase ELISA (Leinco Technologies, Inc., R&D systems) are designed to measure human resistin in cell culture supernates, serum and plasma. rhRES and anti-rhRES antibodies are used in sandwich enzyme immunoassay techniques to accurately quantify hRES using these recombinant factors; that is, these assays produce linear curves similar to the standard curves obtained using the rhRES standards. Other advantages of using recombinant generated proteins include not only their availability but also their purity, homogeneity, and processing. Thus, rhRES and anti-rhRES antibody provide the essential components from which to develop a qualitative recombinant-based RES-MSIA for determining concentration values of endogenous human resistin from plasma.

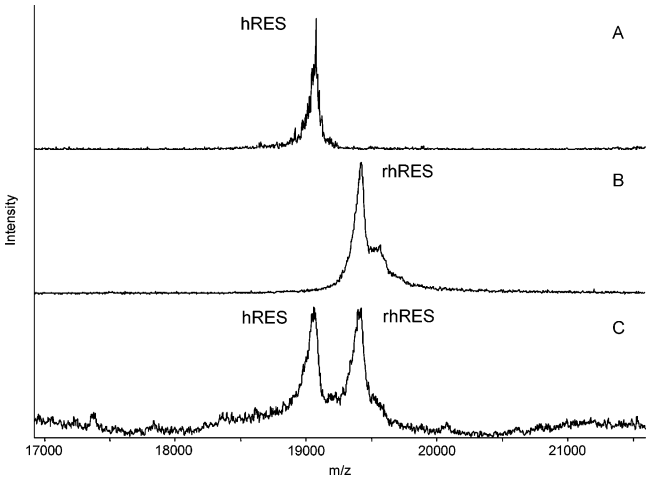


**Figure 3.** Results of anti-rhRES MSIA. RES-MSIA spectra of (a) human plasma, (b) recombinant human resistin (rhRES, 8 ng/mL) in HBS-EP, and (c) mixture of human plasma and rhRES (8 ng/mL). Signals at $m/z$ = 19 103 and 19 452 are observed, corresponding to human RES (hRES, $m/z$ = 19 104) and recombinant RES (rhRES, $m/z$ = 19 454), respectively.

Processed endogenous human resistin is a disulfide-linked homodimer of the 90-amino acid residue monomer with an average mass of 19 104 Da. rhRES is obtained from *E. coli* as a purified 19 454-Da dimeric protein that consists of two 92-amino acid polypeptide chains joined by a disulfide bond. These mass differences, in conjunction with anti-RES immunoaffinity to both rhRES and endogenous proteins, provide unique features from which to develop a recombinant-based RES-MSIA. Figure 3 shows RES-MSIA analysis of previously frozen human plasma, HBS buffer spiked with rhRES (8 ng/mL), and rhRES (8 ng/mL) spiked plasma. The three spectra show strong signals from both human and recombinant RES, labeled hRES (Figure 3a), and rhRES (Figure 3b), respectively. Figure 3c displays the combined immunoaffinity isolation of the RES analogues with fully resolved signals from hRES and rhRES, a characteristic critically important for quantitative RES-MSIA.

**Generation of RES-MSIA Working Curve.** Recombinant-based RES-MSIA makes use of both readily available rhRES and anti-rhRES antibody cross-reactivity to both recombinant and endogenous proteins for combined immunoaffinity capture and MALDI analysis. A quantitative working curve was generated by combining endogenous hRES from a normal human plasma sample with rhRES at known concentrations. Six standard samples from the previously described serial dilution series were used to construct the RES-MSIA working curve. As shown in Figure 4, representative results of immunoaffinity-isolated endogenous hRES with increasing concentrations of recombinant human resistin are displayed in a stacked overlay spectral view. RhRES standards normalized to hRES, generate the normalized integral values ($I_{rhRES}/I_{wt}$) of the working curve, shown in Figure 5, covering a rhRES concentration range of 2.5−80 ng/mL. The curve shows good linearity with linear regression data fitting yielding the relationship, $I_{rhRES}/I_{hRES} = 0.1244$ [rhRES (ng/mL)] − 0.1562 ($R^2 = 0.9982$, SD = 0.174). These results indicate that both the antibody-based retrieval and MALDI-MS analysis per-

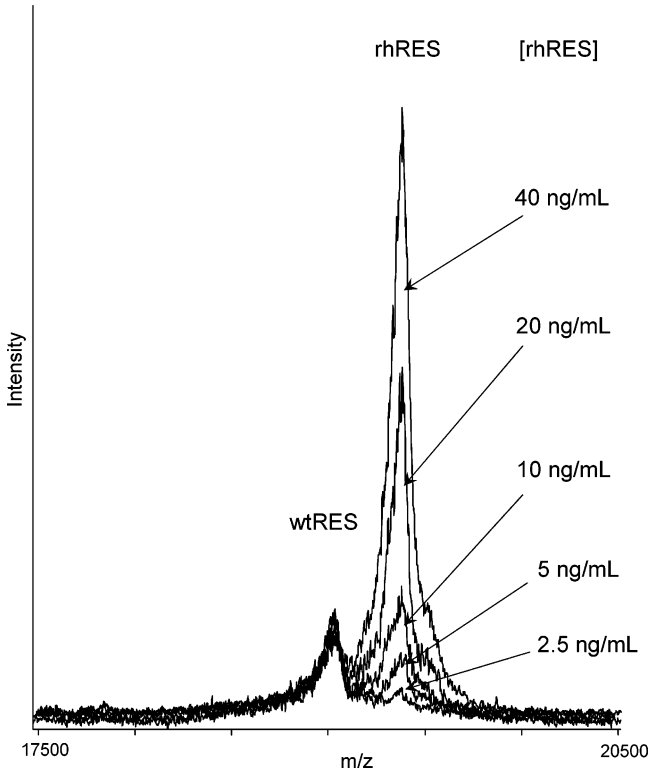


**Figure 4.** Representative RES-MSIA standard addition stacked spectra showing representative spectra of data used to generate the working curve. Recombinant human RES concentrations of 2.5−40 ng/mL were investigated. Human RES from the endogenous plasma sample was used as an internal reference standard for *y*-axis normalization.

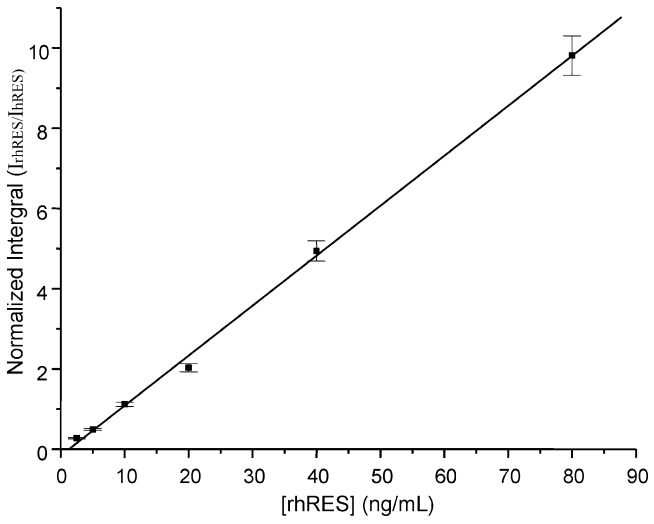


**Figure 5.** MSIA working curve constructed using data represented in Figure 4. The 32-fold range was spanned with good linearity ($R^2$ = 0.9982). Error bars reflect the standard deviation of 5 repetitive 200-laser shot spectra acquired from each sample target spot.

formed below saturation and that adequate resolution was observed between the rhRES and hRES signals in the absence of interference from any other biomolecules during affinity capture.

**Quantification of RES in Human Plasma.** Plasma samples from nine normal subjects and four cTnI positive cardiac subjects were analyzed as a mock population study from which to assess the ability of the RES-MSIA to identify rhRES and hRES quantitative variability as well as levels of endogenous RES within the

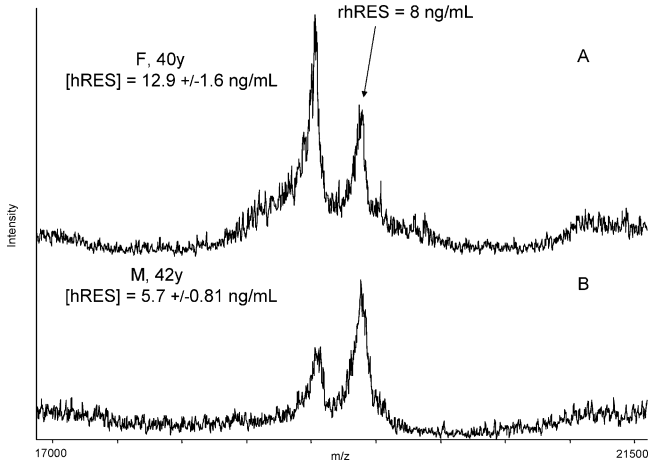


**Figure 6.** Representative spectra of RES-MSIA applied to normal samples displaying the putative difference of hRES between genders. rhRES at 8 ng/mL was used as the internal reference standard. (A) Effective retrieval of hRES from a 40-year-old female donor. (B) hRES from a 42-year-old male donor.

**Table 1. Application of RES-MSIA to Normal and Cardiac Samples**

| | | | [RES] average (ng/mL) | | |
|---|---|---|---|---|---|
| TnI (ng/mL) | gender | age | MSIA | rel ratio | ELISA |
| Normal | | | | | |
| | M | 23 | 8.7 ± 0.84 | 7.4 | |
| | F | 29 | 9.7 ± 0.60 | 8.4 | 7.5 |
| | M | 29 | 9.3 ± 0.00 | 8.0 | 8.3 |
| | F | 40 | 12.9 ± 1.63 | 11.5 | |
| | F | 40 | 14.6 ± 2.66 | 13.3 | |
| | M | 42 | 5.7 ± 0.81 | 4.5 | 5.5 |
| | F | 46 | 9.2 ± 1.79 | 7.9 | |
| | M | 51 | 9.1 ± 1.40 | 7.8 | |
| | F | 65 | 10.0 ± 0.78 | 8.7 | |
| Cardiac | | | | | |
| 1.5 | M | 70 | 33.1 ± 2.44 | 31.7 | 29.0 |
| 1.7 | F | 65 | 43.0 ± 2.20 | 41.5 | 31.8 |
| 1.9 | M | 63 | 13.8 ± 1.13 | 14.7 | |
| 2.3 | M | 61 | 24.6 ± 4.03 | 27.5 | |

cohort. Sample preparation, extraction, rinses, and elution steps were performed identical to prior analyses. Using a multichannel pippeter, the total analysis required ~1 h to perform. Figure 6 shows two representative spectra resulting from RES-MSIA analysis of two normal plasma samples using rhRES at 8 ng/mL as the internal reference standard. Quantified hRES values are displayed for the two analyses (5.7 ± 0.68 ng/mL, Figure 6A and 12.9 ± 1.63 ng/mL, Figure 6B); the combined immunoaffiintiy isolation displays favorably resolved signals of hRES from rhRES.

Information regarding subject gender, age, and select representative ELISA results (obtained from R&D systems) from application of RES-MSIA to normal and select cardiac subject analysis are shown in Table 1. Column four, under the MSIA header, depicts the resulting hRES concentrations (ng/mL) for the cohort obtained using the MSIA working curve line equation. Column five in Table 1 shows the concentration of hRES (ng/mL) in each of the plasma samples utilizing a rapid ancillary relative ratio method. Here, the concentrations of hRES for the normal cohort were determined from the relative ratio of rhRES (at 8 ng/mL) to relative intensity versus hRES relative intensity,

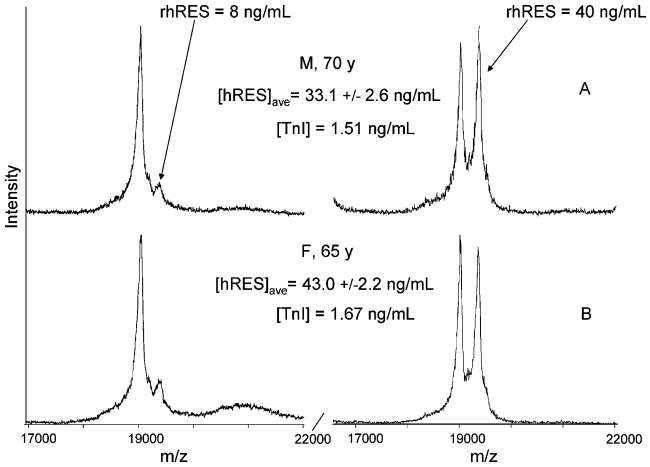


**Figure 7.** Representative results of RES-MSIA applied to cardiac samples stratified for cTnI. rhRES at both 8 and 40 ng/mL were used as the internal reference standard. Trace A and trace B show representative spectra and effective hRES retrieval from two donor samples, a 70-year-old male and a 65-year-old female.

i.e., [hRES] = [rhRES]/Rel $Int_{rhRES}$ × Rel $Int_{hRES}$. Mean resistin concentration in normal samples (9.9 ng/mL, range 5.7−14.6) is in relative agreement within the two metrics, the external ELISA values, as well as other studies determined by other techniques.[24] Normal plasma resistin levels (median [range], ng/mL) were modestly higher in women than men (11.3 [9.2−14.6] verus 8.2 [5.7−9.3] ng/mL, respectively).

RES-MSIA was also performed on small cardiac cohort prequantified (AxSYM) for cTnI utilizing the IRS, rhRES, at both 8 and 40 ng/mL (Table 1). Figure 7, traces A and B (stratified for cTnI at 1.5 and 1.67 ng/mL, respectively), show the representative resistin spectra that resulted in an averaged hRES value of 33.1 and 43 ng/mL, respectively. These values are somewhat higher than the 29 and 31.83 ng/mL values obtained externally by ELISA. The cTnI cardiac positive samples show increased hRES levels in virtually all of the samples (generally a 3-fold increase of hRES from normal levels), demonstrating a mean of 28.6 ng/mL and a range of 13.8−43 ng/mL and underscoring an enhanced level for the female donor over the male donor samples. The average concentration of hRES was also determined by a relative ratio method, shown in Table 1 as a separate column; these values are comparable to the RES-MSIA values and similar to the select ELISA values. Overall, these enhanced levels of hRES imply inflammatory effects in these cardiac positive samples. The RES protein, viewed as a single protein (dimer) without truncations or posttranslational modifications from which to identify digitally distinct frequencies, appears to be indicating cardiac inflammation in conjunction with the diseased state. A more comprehensive understanding will require use of additional samples and contributing biomarkers in panel assays from which to gain combined diseased state indicator frequencies.

## CONCLUSION

Integrating immunoaffinity capture and MALDI-TOF MS, the MSIA approach provides rationally defined qualitative protein detection and identification based upon the inherent mass signature(s) of the target analyte(s) as well as quantitative analysis in which normalization of the endogenous protein spectra to an internal reference standard combined with absolute concentration assignment from a standard addition curve. Transposition to recombinant proteins as standards and immunoconjugates extends the scope of protein targets and biomarkers available for MSIA development and cohort analysis. In the current study, we used the recombinant-based RES-MSIA approach for affinity isolation and qualification of endogenous and recombinant resistin from human plasma as well as mass spectrometric quantification of the RES species. The recombinant-based RES-MSIA analyses of rhRES standards, using normal human plasma internal reference, generated a working curve from which normal and cardiovascular stratified human plasma samples were quantified. Normal human plasma RES levels ranged from 5.7 to 14.6 ng/mL and showed slightly higher levels in normal female samples than male, in accordance with what has been observed using other techniques (ELISA) for normal as well as diabetic sample analyses.[27,44] Cardiac plasma investigation generally demonstrated a 2−3-fold elevations over normal for cTnI positive (coronary event ≥0.4 ng/mL[45]) samples. The recombinant-based MSIA may be used with either polyclonal or monoclonal antibody, does not require extensive selection of internal reference standards, i.e., from different species or chemically modified variants, and requires that the recombinant analogue is readily available and of an addressable mass difference from the endogenous protein. The development of a recombinant-based RES-MSIA has not only shown the viability of using a recombinant protein whose inherent availability, purity, homogeneity, and mass difference from wild type and immunoaffinty provide the essential components for the RES-MSIA development, but also enabled the application to mass spectrometric qualification and subsequent quantification of the target biomarker, hRES, at low-nanomolar (~5−30 ng/mL) concentrations in normal and cardiac stratified human plasma samples.

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