Multiplexed Mass Spectrometric Immunoassay in Biomarker Research: A Novel Approach to the Determination of a Myocardial Infarct

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Reported here is the development of a multiplexed mass spectrometric immunoassay (MSIA) for the detection of myocardial infarction (MI). The assay is the product of a study that systematically progresses from biomarker discovery—to identification and verification—to assay design, data analysis, and statistical challenge. During targeted population proteomics investigations, two novel biomarkers, serum amyloid A1 and S-sulfated transthyretin, were found to be responsive to MI. These putative markers were subsequently screened in larger cohorts of individuals to verify their responsiveness toward MI. Upon verification, a multiplexed assay was designed that was capable of simultaneously monitoring the new markers plus a previously established MI-marker (myoglobin). The multiplexed MSIA was applied to two 96-sample sets comprised of 48-MI/48-healthy and 19-MI/77-healthy, which served as training and case cohorts, respectively. Data evaluation using either preset reference levels or multivariate analysis exhibited sensitivities and specificities of >97%. These findings illustrate the importance of using systematic approaches in clinical proteomics to discover biomarkers and produce high-performance assays relevant to disease.

Keywords: mass spectrometry • immunoassay • myocardial infarction • multiplexing • human plasma

Introduction

A multiple-marker mass spectrometric immunoassay (MSIA) panel tailored for the determination of a disease or disorder is of special interest because a single analyte is unlikely to provide optimal specificity and sensitivity. Many clinical outcomes are multidimensional, and a single measure may miss domains of interest, and further, it is very difficult to capture both benefit and harm within a single measurement. Panels (or composite) of biomarkers are therefore the direction that clinical chemistry will most likely follow into the future. In this study, we put this principle into practice and set out to develop a novel multiplexed MSIA that could be used to determine a myocardial infarction (MI). We selected MI because: (1) biochemical testing plays a key role in its diagnosis, (2) MI is a common event with considerable morbidity and mortality, and (3) the established markers, even when employed in a panel, are considered by some to be sub-optimal. We therefore view studies of MI as an ideal model upon which to develop targeted clinical proteomics assays.

The approach taken in these studies consisted of several steps devoted to ultimately producing a single MSIA able to simultaneously analyze multiple known or newly discovered biomarkers. Categorically, these steps were:

1. Preliminary Screening. In this operation, proteins from plasma samples of diseased individuals were analyzed using individual mass spectrometric immunoassays.1 As presented here, 25 full-length protein assays were performed as described previously.2 Ten plasma samples (MI-affected) were screened, and the results viewed for qualitative and quantitative differences relative to data established during the screening of a larger, healthy population. It should be noted that it was not the intent of the preliminary screening to validate biomarkers to any statistical extent; rather the intent of the prescreening was to rapidly denote overt differences in proteins that may occur in the diseased samples. As such, a relatively small sample cohort was the subject of prescreening operations, with progressively larger cohorts reserved for screening once putative biomarkers were identified.

2. Identification of Putative Biomarkers. During this step, protein species that were (tentatively) found able to discriminate between the disease cohort and the healthy population were identified (structurally characterized) using analytical approaches classically used in biomolecular mass spectrometry. The resulting identity of the putative biomarkers served as the starting point for more in-depth “verification” studies.

3. Verification. The intent of this operation was to more formally challenge the putative biomarkers by establishing their behavior in larger cohorts. Cohorts consisting of equal numbers of diseased and healthy samples (i.e., 48 of each) were screened using single-analyte MSIA’s that targeted the putative biomarkers. Data produced from these assays were then evaluated for consistent qualitative/quantitative differences able to separate the cohorts into different classes with a statistical degree...
of certainty. We view this “verification” process as a means of independently evaluating each putative biomarker before it is amalgamated into a multiplexed assay.

4. Knowledge Assembly and Next-Generation Assay Design. Under this step, knowledge was assembled to develop next-generation multiplexed assays. Notably, such knowledge can stem from any of number of sources—e.g., proteomics-based discovery or well-founded knowledge from past clinical investigations. Such is the case here, where an assay was designed based on a combination of new findings (from Steps 1–3) and a clinically established MI marker (myoglobin). Important to designing such assays is compatibility in analysis—i.e., that the target proteins are extracted and analyzed using a single methodology. This criterion ultimately imposes some technical limitations stemming from different conditions used in the mass analysis of disparate markers or the ability to develop extraction devices and conditions able to efficiently extract multiple proteins at largely differing concentrations. However, given a fundamental understanding of immunoaffinity isolation, the MALDI process and TOF instrumentation, markers can be judiciously grouped into single assays that stand a reasonable chance of success in analysis. (For instance, different matrices and instrument settings are typically used for different mass ranges during MALDI-TOFMS. This need for different analysis conditions is the reason that two other MI markers—creatinine kinase MB and cardiac troponin—were not included in the final assay developed during this study.)

5. Data Generation and Analysis—Equal Number Cohorts. This step was devoted to determining and critiquing data acquisition and evaluation methodologies. Plasma samples from two cohorts of moderate and equal size (healthy and MI-affected; 48-samples each; age and sex matched) were analyzed in parallel using the assay and devices developed during Step 4. The resulting data was then evaluated using more than one approach. Reference-level methods were applied to each marker (either individually or in various combinations) as well as more sophisticated machine learning approaches. The outcome of this step was the final (start-to-finish) assay and data evaluation method to be used in blind and randomized challenge.

6. Blind and Randomized Challenge of Final Assay. The intent of this step was to test the final multiplexed assay and data evaluation method as a prelude to longer-term application in clinical investigations. The final multiplexed assay was applied in the blind and randomized analysis of plasma samples from 77 healthy individuals and 19 MI-affected individuals randomized in a 96-well format with no a priori knowledge of the disposition of the sample in each well. This sample set was intentionally constructed to more accurately reflect the natural frequency of MI in the general population. The outcome of this challenge was simply to evaluate whether the assay exhibited any statistical merit in detecting disease in a sample population reflective of that which may be encountered in real-world application. If poor performance was observed, the assay may be improved (e.g., by addition of other markers) or simply abandoned. Given positive results, the assay can be moved forward into more regimented clinical studies devoted to, e.g., investigating larger cohorts, challenging the assay with alternate disease and tracking assay behavior longitudinally.

Taken collectively, we believe these steps represent a progressive and systematic philosophy to biomarker discovery—through—application using targeted proteomics-based technologies. Given below, we demonstrate the process and discuss the potential of this strategy through the example of myocardial infarction.

Experimental Procedure

Preliminary Screening, Biomarker Identification, and Verification. Plasma samples from ten MI patients were screened for select proteins in a Population Proteomics analysis, as described in Reference 2. Data resulting from these assays were evaluated manually to determine comparative differences relative to the foundational data given in the same reference. Noticeable qualitative (i.e., variants) and semiquantitative differences were observed in two profiles resulting from the targeting of transthyretin (TTR) and serum amyloid A (SAA).4 The identities of the exact determinant species was confirmed using mass mapping and chemical treatment as described in refs 2, 3, and 4. Verification assays for these two proteins were performed on 48 MI samples versus 48 healthy controls. TTR assays were performed as described in Reference 3. SAA assays were performed as described in ref 4, with the exception of adding 30 μL of equine serum to each sample, containing equine SAA (eSAA; M, = 12 289), which was co-extracted and analyzed simultaneous to the human SAA to serve as an internal reference standard for relative quantification.5–8 Two data sets were produced, and ion signals from each set, sulfonated (SS) -TTR and cysteinylated (cys) -TTR, and, hSAA1α and eSAA, were baseline integrated over a mass range of 0.15% of the M, of each species using Proteome Analyzer Software (Intrinsic Bioprobes, Tempe, AZ). For comparison, the integrals for SS-TTR and hSAA1α were normalized to their respective internal references (cysteinylated TTR and eSAA) and the values plotted with respect to the health state of the individual.

Multiplexed Mass Spectrometric Immunoassay. Extraction devices for the multiplexed mass spectrometric immunoassay were prepared by coupling a mixture of mouse anti-human monoclonal antibody to serum amyloid A (MO–C4002BA; Anogen, Mississauga, ON, Canada), rabbit anti-human polyclonal antibody to transferrin (A0002; DakoCytomation, Carpinteria, CA), and goat anti-human polyclonal antibody to myoglobin (70-MG60; Fitzgerald, Concord, MA), to CD1 (1,1'-Carbonyldimidazole)- activated affinity-pipets using protocols previously described.19 In previous studies, we have found that these antibodies work well in MSIA application and are able to detect microheterogeneity in the three target proteins.10,11 Through side experiments, it was determined that a mixture of antibodies at the ratio of 0.08:0.04:0.10 mg/mL (SAA:TTR: MYO) was able to detect SAA and TTR at basal concentrations, and MYO at concentrations above 100 ng/mL. Using the extraction devices, each sample set was processed in parallel using a Beckman Multimek Automated 96-Channel Pipettor (Beckman Coulter, Fullerton, CA). The protein extraction/affinity capture process followed established protocols that have been published previously.3,12–14 Briefly, multiplexed affinity pipets were mounted onto the head of the Multimek pipettor and initially rinsed with 100 μL of HBS buffer (10 cycles, each cycle consisting of a single aspiration and dispense through the affinity pipet). Next, the pipets were immersed into the sample tray, and 150 aspirations and dispense cycles (100 μL volumes each) were performed, allowing for affinity capture of the targeted protein. Following affinity capture, the pipets were rinsed with HBS (10 cycles), water (10 cycles), 2 M ammonium acetate–acetonitrile (4:1 v/v) mixture (10 cycles), and two final water rinses (10 cycles each). The affinity pipets
Results and Discussion

Samples. Heparinized human plasma samples (in 2–5 mL volumes) were obtained through ProMedDX (Norton, MA). Disease samples were collected from symptomatic individuals who arrived at emergency rooms and were subsequently admitted and treated for MI. All samples had elevated levels of CK-MB (> 21 µg/L). Information regarding the time of blood draw, e.g., time-from-onset (chest pain) or length of wait in the emergency room was unavailable. Age and sex matched healthy controls were also obtained for each sample set. The samples were collected at certified blood donor and medical centers and provided labeled only with a barcode and an accompanying specification sheet containing information about the gender, age, and ethnicity of each donor, thus ensuring proper privacy protection. All plasma samples were determined nonreactive for common blood infections and were kept at ~75 °C until use. Due to recent changes in HIPAA regulations, information regarding race was no longer available for all samples and therefore was not utilized in the sample selection criteria of this study.

Biomarker Discovery, Identification, and Verification. Previously, we have reported on technologies and methodologies able to characterize full-length plasma proteins for the purpose of determining differences (e.g., identifying variants and quantitative modulations) found among the general population. In furthering this approach, we have developed 25 individual assays that have been incorporated into a high-throughput screening platform and used in creating a data foundation (in the healthy population) against which results from disease cohorts can be compared. The same panel of assays was applied to a small number of MI patients for the purpose of screening for putative markers. Plasma samples from ten MI-affected individuals were screened on a per protein basis to determine qualitative and/or quantitative differences away from foundational data. During this exercise, two plasma proteins, serum amyloid A and transthyretin, were observed to differ notably in the MI cohort. Variants of these proteins were subsequently identified and then “verified” as putative markers for AMI using a secondary screen in which 48 MI samples were compared to 48 healthy samples.

Serum amyloid A (SAA) describes a family of three genes found in humans (SAA1, SAA2 and SAA4), which produces proteins that act as apolipoproteins by chaperoning in the transport of high-density lipoprotein particles. During “acute phase response”, the body’s reaction to the immediate onset of inflammatory stimulus with the purpose of counteracting the challenges of tissue injury, infection and trauma, SAA1 and SAA2 levels in plasma have been shown to increase as much as 1000-fold from basal concentration of ~1–5 mg/L. Accordingly, low-levels of SAA can be used as a general indicator of good health, whereas higher levels may be a particularly useful indicator of chronic or immediate ailments, including MI. Important to the discussions at hand, we have previously noted a high degree of heterogeneity in SAA between individuals. There are at least three different causes of the microheterogeneity; simultaneous determination of both SAA1 and SAA2, variably truncated versions of the two major gene products (generated posttranslationally), and point mutations contained in all forms of gene products. During a secondary (“verification”) screen, the intact SAA1 species (104 amino acids; Mᵣ = 11 683) was found to be preferentially elevated in MI individuals versus the healthy controls (See Figure 1A). On the basis of these observations, SAA1 was chosen as a putative marker to be included in a multiplex assay.

Transthyretin (TTR) is a noncovalently associated tetrameric protein, commonly found in serum and cerebral spinal fluid. Functionally, TTR serves unaccompanied in the transport of thyroid hormones or in complexes with other proteins in the transport of various biologically active compounds. Structurally, the wild-type (wt) TTR subunit is comprised of 127 amino acids and has a Mᵣ of 13 762. Previous studies suggest that plasma proteins such as TTR are able to undergo chemical modification induced by oxidative stress, as might be expected during negative cardiac events. Importantly, TTR has been studied extensively by mass spectrometry resulting in numerous publications devoted to the characterization of TTR variants on both the point mutation and posttranslational modification levels. During preliminary screening, a variant tentatively identified as S-sulfonated TTR (SS-TTR) was observed at high levels and at a notably high frequency relative to the control population. Subsequently, mass mapping combined with chemical reduction and high performance mass spectrometry was used to confirm the site of S-sulfonation as Cys10, which is consistent with findings in other laboratories utilizing similar approaches. During the “verification” screen, the SS-TTR variant was observed at elevated levels in the MI samples (See Figure 1B). Given its high frequency of occurrence at elevated levels within the cardiac population, SS-TTR was included for monitoring as part of a multiplexed assay.

The third protein incorporated into the multiplex assay was myoglobin. Myoglobin (MYO) is a globular heme-producing protein found in the cytoplasm of skeletal and cardiac muscle cells. Due to its small size (153 amino acids; Mᵣ = 17 053) and storage location within the cell, MYO is quickly released from necrotic myocytes – most pronounced during tissue damage stress. This phenomenon results in a temporal release that can be observed during MI, with elevated levels becoming detectable within 2 h of onset and reach maximum levels at 6–8 h. This quick release makes MYO one of the earliest markers for MI assessment, and accordingly, it has been used as an FDA-
regulated MI diagnostic assay since the late 1990s (FDA: CDHR Listing Database, #866.5680).

Development of Tri-Marker Assay. On the basis of the preliminary findings, a multiplexed MSIA was designed to target the two new putative biomarkers, SAA1α and SS-TTR, as well as the established MI marker myoglobin. The assay relied on technologies as previously described1,6 and was designed empirically by systematically varying the ratio of antibodies targeting the markers. The resulting assay was able to detect SAA1α and TTR at basal (healthy) levels, however, was only able detect myoglobin at levels above the clinical reference level (100 ± 20 µg/L as determined by side experiments). Thus, the assay automatically imposed a cutoff value for myoglobin equal to a physiological level commonly used for MI assessment with other analytical platforms,22 thereby taking full advantage of past clinical findings relating myoglobin levels with myocardial infarction.

Application of Tri-Marker Assay. Two sample sets were investigated in the course of study. The first set of 96-samples was comprised of an equal number of healthy and MI-affected individuals (termed 48/48). The second study set of 96-samples was designed to more accurately reflect the natural occurrence of cardiovascular disease in the general population (19/77; MI/Healthy). Spectra resulting from the use of the tri-marker assay were found to differ both qualitatively and semiquantitatively dependent on the individual under analysis. The extremes of these differences are shown in Figure 2, which shows spectra obtained from a (A) healthy and (B) an MI-affected individual. Figure 3 shows a three-coordinate map of the relative integrals of SAA1α, SS-TTR, and MYO determined for the 192-samples under study. Notably, control samples from both sets (red circles and squares) are observed to cluster at relatively low values for two of the three markers, SS-TTR and MYO. Outliers from this cluster are observed predominantly for SAA1α values, however, clearly not to the degree observed for the MI samples (blue circles and squares).

Data Evaluation Method 1—Independent Markers with Analytical Reference Levels. In the first data evaluation method, the 48/48 sample set was viewed as a training set in order to define relative integrals that could be used as reference levels for each of the three markers. In this process, each marker was treated individually to find an analytical reference level that equally balanced the number of false positives with

Figure 1. Relative integrals of SAA1α and SS-TTR for healthy and MI individuals. (A) The average value for SAA1α is observed to increase from 6.22 (s.d. = 12.8; n=48) to 50.4 (s.d. = 108; n = 48) for the healthy versus MI individuals, respectively. (B) The average value for SS-TTR is observed to increase from 0.037 (s.d. = 0.026; n=48) to 0.38 (s.d. = 0.319; n = 48) for the healthy versus MI individuals.

Figure 2. Spectra obtained from healthy and MI-affected samples. (A) Healthy individual where (I) des R–SAA1α, (II) SAA1α, (III) TTR and (IV) cys-TTR are observed. (B) MI-affected individual where SAA1α, (V) SS-TTR, and (VI) MYO are observed at elevated levels. Note that the spectra are normalized to cys-TTR, which was found present in all spectra and was thus used as an internal reference standard (i.e., relative integral = 1.00). Relative integrals (of the markers) for all samples are given in the Supplementary Tables 1 and 2 (see Supporting Information) and shown in Figure 3.

the number of false negatives. This simple methodology was used to gauge the relative increase (if any) in predictive value that stems from the cooperative use of the three independent markers. Table 1 gives reference values determined from this
exercise. Because of the original methodology used, i.e., balancing the number of false readings when determining the threshold values, it is expected that all metrics will decrease when criteria require the presence of any one marker and another, or the presence of all three of the markers. Such is the case, as observed when the metrics for (SAA and TTR), (SAA and MYO), (TTR and MYO), (SAA and TTR and MYO) are compared with their individual contributors. However, the predictive metrics are observed to increase when all combinations of two-marker assays are considered cooperatively, i.e., when (SAA and TTR), or (SAA and MYO), or (TTR and MYO) are used for classification. These results are viewed as encouraging in the use of the SAA/TTR/MYO tri-marker assay in the determination of MI and indicate the use of the cooperative two-marker approach in making MI classifications.

Previous suggestions/criticisms have been directed at the usefulness of clinical proteomics approaches when data from equally balanced sample sets are extrapolated (for diagnostic application to sample sets) proportioned to more accurately reflect the natural occurrence of disease states within populations. To address this issue, a second set of samples, proportioned 19/77 (MI/Healthy), was analyzed in order to challenge the tri-marker assay as applied to a general population that may require a point-of-care assay. All methodologies used in the 48/48 sample set remained the same with the exception of performing the analyses in a blinded and randomized manner. Only after analysis and classification were the true classifications of the sample revealed for correlation. Table 2 shows the results of the exercise. When the cooperative two-marker approach was used, only two of the 96 samples were classified incorrectly (as false positive), yielding results comparable to those of the training set. However, a number of metrics – notably, the predictive values of the individual, tandem or all three markers – are observed to deteriorate in the 19/77 sample set. This phenomenon is in agreement with the arguments posed in ref 23, which summarizes that good clinical statistics based on equal number comparisons may deteriorate when applied to skewed sample sets.

Summarily, these results emphasize the use of multiple markers in cooperation to increase the predictive value of this assay. However, when using this evaluation method all of the markers were weighted equally, i.e., each yielded a yes/no answer based on whether it was above/below a reference level. As such, the evaluation method makes no provision for weighting the importance of each marker relative to MI and may therefore introduce error into the results by giving a marker slightly more or less credit than it deserves. Accordingly, a more sophisticated multivariate approach (able to weight variables) was investigated in an attempt to reach higher levels of performance.

Data Evaluation Method 2—Random Forest Classification.

Data were subjected to a second evaluation using random forest classification. Overviews of the approach as applied to mass spectrometry data are given in reviews authored by Wu et al.24 and Izmirlian.25 As used here, the methodology of Wu et al was applied to data given in the Supplementary Tables 1 and 2 (see Supporting Information). Foremost, the 48/48 data set was evaluated for potential error rate. The data set was randomly split into a training set of 60 spectra and a challenge set of 36 spectra. The training data was used to build classification rules, from which the challenge data could be used to estimate error rates. In all, the procedure was repeated 100 times resulting in 3534 correct classifications for the 3600 challenges, which suggests an error rate of ~1.8%. Subsequently, the 48/48 data set was used as a training set with the 19/77 data set used as a challenge set (Table 3). A single false positive was observed, yielding a sensitivity and specificity of 100% and 98.7%, respectively.

Conclusion

Given here is a systematic study designed to ultimately produce a multiplexed mass spectrometric immunoassay for the detection of disease. The study can be broken into several integral components—screening/ discovery, identification, verification, knowledge assembly, assay design, data analysis, and challenge—each of which plays an important role in the overall process. The first three components resulted in the independent discovery and identification of putative biomarkers, i.e., differences were first recognized during screening and subsequently identified using mass spectrometry-based analytical methodologies. The resulting newly discovered knowledge was then verified on a larger scale and combined with existing knowledge, derived largely from pre-proteomics-era clinical efforts, to design a multiplexed assay that specifically targeted the markers.

In the given example, the three markers chosen for assay design were determined to be responsive to MI as part of detached and independent studies. Thus, each marker is known to exhibit predictive qualities toward MI prior to its inclusion into the multiplexed assay. Once included into the assay, the merits of each species can be evaluated individually as well as when used in combination with the other markers. As shown here, the results of independent data evaluation re-establish each of the markers as having its own merit toward determining MI and, moreover, the performance of the assay is observed to increase when the markers are used in cooperation. Analysis of the same data using more sophisticated multivariate analysis further illustrates this cooperative effect. Collectively, these
adding to the current knowledge and application of 
proteomics. Fittingly, Anderson et al has recently 
emphasized the importance of proteomics in this role and has 
proposed new techniques. In this manner, the approach is in 
good standing with research articles that evaluate technologies and 
findings based on newer methods, our results indicate maximum sensitivity of 100%, 
and/or in the detection of MI. 
observations support the use of the three markers as part of a 
panel responsive to MI. 

On this closing note, the biomarkers and assay described in 
this research yielded predictive metrics that exceed those found 
using conventional MI biomarker assays and with further 
refinement and validation (e.g., longitudinal studies, 
application to larger cohorts and challenge against other diseases) may 
very well have application in the field of cardiovascular research and/or in 
the detection of MI. [Using two data evaluation methods, our results indicate maximum sensitivity of 100%, 
and specificity of 98.7%, PPV of 95%, and NPV 100%. Conventionally, CKMB, cTn, and MYO are used in the 
determination of MI. The maximum reported values of these biomarkers are 
taken from refs 26, 27, and 28] CKMB: sensitivity = 99%, 
specificity = 89%, PPV = 64%, NPV = 99.8%; Troponin I: sensitivity = 33.3%, specificity = 99.4%, PPV = 78.6%, NPV = 96.6%; MYO: sensitivity = 84.6%, specificity = 71.1%, PPV = 20.4%, NPV = 98.3.] From an historical perspective, the assay can be viewed in its simplest form as an improved myoglobin assay. In this manner, the approach is in good standing with currently applied clinical and diagnostic knowledge, as well as 
is responsive to technologies and findings based on newer proteomics techniques. Fittingly, Anderson et al has recently 
emphasized the importance of proteomics in this role and has 


Table 1. Results from Reference Level Evaluation of 48/48 (MI/Healthy) Sample Set

<table>
<thead>
<tr>
<th>marker</th>
<th>sensitivity</th>
<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>relative integral value determined for threshold</th>
<th>false readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA</td>
<td>91.7</td>
<td>91.7</td>
<td>91.7</td>
<td>91.7</td>
<td>1.35</td>
<td>FP = 4, FN = 4</td>
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<tr>
<td>SSTTR</td>
<td>95.8</td>
<td>97.9</td>
<td>97.8</td>
<td>95.9</td>
<td>0.45</td>
<td>FP = 1, FN = 2</td>
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<tr>
<td>MYO</td>
<td>87.5</td>
<td>87.5</td>
<td>87.5</td>
<td>87.5</td>
<td>0.02</td>
<td>FP = 6, FN = 6</td>
</tr>
<tr>
<td>(S and T)</td>
<td>87.5</td>
<td>89.6</td>
<td>89.4</td>
<td>87.7</td>
<td>same</td>
<td>FP = 5, FN = 6</td>
</tr>
<tr>
<td>(S and M)</td>
<td>79.2</td>
<td>79.2</td>
<td>79.2</td>
<td>79.2</td>
<td>same</td>
<td>FP = 10, FN = 10</td>
</tr>
<tr>
<td>(T and M)</td>
<td>83.3</td>
<td>85.4</td>
<td>85.1</td>
<td>83.6</td>
<td>same</td>
<td>FP = 7, FN = 8</td>
</tr>
<tr>
<td>All three</td>
<td>75.0</td>
<td>77.1</td>
<td>76.5</td>
<td>75.5</td>
<td>same</td>
<td>FP = 11, FN = 12</td>
</tr>
<tr>
<td>(S and T) or (S and M) or (T and M)</td>
<td>97.9</td>
<td>100</td>
<td>100</td>
<td>98.0</td>
<td>same</td>
<td>FP = 1, FN = 0</td>
</tr>
</tbody>
</table>

* The sample set consisted of 48 MI-affected and 48 healthy individuals, with mean ages of 52 (range: 21-66) and 51 (range: 21-66), respectively.

Table 2. Results of Reference Level Evaluation of 19/77 (MI/Healthy) Sample Set

<table>
<thead>
<tr>
<th>requirement (marker above threshold)</th>
<th>sens</th>
<th>spec</th>
<th>PPV</th>
<th>NPV</th>
<th>relative integral value used as reference level in classification</th>
<th>false readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA</td>
<td>89.5</td>
<td>80.5</td>
<td>53.1</td>
<td>96.8</td>
<td>1.35</td>
<td>FP = 15, FN = 2</td>
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<tr>
<td>SSTTR</td>
<td>94.7</td>
<td>100</td>
<td>100</td>
<td>98.7</td>
<td>0.45</td>
<td>FP = 0, FN = 1</td>
</tr>
<tr>
<td>MYO</td>
<td>89.5</td>
<td>81.8</td>
<td>54.8</td>
<td>96.9</td>
<td>0.02</td>
<td>FP = 14, FN = 2</td>
</tr>
<tr>
<td>(S and T)</td>
<td>84.2</td>
<td>80.5</td>
<td>51.6</td>
<td>95.4</td>
<td>same</td>
<td>FP = 15, FN = 3</td>
</tr>
<tr>
<td>(S and M)</td>
<td>78.9</td>
<td>58.4</td>
<td>34.0</td>
<td>91.8</td>
<td>same</td>
<td>FP = 29, FN = 4</td>
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<tr>
<td>(T and M)</td>
<td>84.2</td>
<td>81.8</td>
<td>53.3</td>
<td>95.4</td>
<td>same</td>
<td>FP = 14, FN = 3</td>
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<tr>
<td>all three</td>
<td>73.7</td>
<td>58.4</td>
<td>32.6</td>
<td>90.0</td>
<td>same</td>
<td>FP = 29, FN = 5</td>
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<tr>
<td>(S and T) or (S and M) or (T and M)</td>
<td>100</td>
<td>97.5</td>
<td>89.5</td>
<td>100</td>
<td>same</td>
<td>FP = 2, FN = 0</td>
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</table>

* The sample set consisted of 19 MI-affected and 77 healthy individuals, with mean ages of 52 (range: 21-66) and 45 (range: 21-69), respectively.

Table 3. Random Forest Classification of Sample Set 17/79

<table>
<thead>
<tr>
<th>requirement</th>
<th>sensitivity</th>
<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>predicted error*</th>
<th>false readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-marker</td>
<td>100</td>
<td>98.7</td>
<td>95.0</td>
<td>100</td>
<td>1.83%</td>
<td>FP = 1, FN = 0</td>
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<td>multivariate</td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Error predicted from the 48/48 training set (3534 correct classifications for 3600 challenges).

foundations upon which to base further studies geared toward the 
stepwise validation and introduction of mass spectrometry-based 
assays into the field of cardiovascular medicine.

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Supporting Information Available: Relative integrals 
for 48/48 and 19/77 data sets. This materials is available free 
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References

research articles


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