Supporting Information Figure 1: Charge deconvoluted mass spectra of apoA1 incubated at room temperature, illustrating the avoidable but significant potential for artifactual protein oxidation due to sample mishandling. Day one is in black, day four is in blue, day twelve is in red, and day sixteen is in green.

Supporting Information

S1 MSIA Methodological Details

S1.1 Materials

Samples from the general U.S. population were collected offsite under IRB approval and, prior to purchase (from ProMedDx, Norton, MA), were publicly available. Plasma samples were received with all personal identifiers removed, allowing the authors to operate under human subjects "Exemption 4" classification pursuant to Federal regulations 45 CFR Part 46.101(b)(4) (Arizona State University IRB Protocol #: 0705001851, originally approved 18 May, 2007). Plasma was stored at -80 ^{o} C until analysis. Carboxydextran functionalized MSIA pipette tips (Cat. No. IBI-CMD-R96) were purchased from Intrinsic Bioprobes, Inc. (Tempe, AZ), now owned by ThermoFisher Scientific. Affinity purified polyclonal goat anti-human apolipoprotein A-I (Cat. No. 11A-G2B) was obtained from Academy Biomedical Co. (Houston, TX) Premixed MES-buffered saline powder packets were obtained from Pierce (Rockford, IL). HEPES buffered saline (10 mM HEPES, 0.15 M NaCl, pH 7.4; a.k.a. HBS-N) was purchased from GE Healthcare Life Sciences. Protein Captrap cartridges for LC/MS were obtained from Michrom Bioresources (Auburn, CA). N, N'- carbonyldiimidazole (CDI) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

S1.2 Immobilization of Antibodies on MSIA Pipette Tips

To prepare MSIA pipette tips for apoA1 antibody immobilization, the unmodified carboxydextran surfaces were rinsed thoroughly with 0.2 M HCl then acetone using a Beckman

Multimek 96 pipetting robot. Tips were subsequently ejected and dried under vacuum. After drying, the tips were activated by exposure to 150 μL of 1,1'-carbonyldiimidazole (50 g/L) in 1-methyl-2-pyrrolidinone for 30 minutes. This was accomplished by repeatedly pipetting (aspirating and dispensing) 100 μL of solution over the tip frits. After two brief rinses in 1-methyl-2-pyrrolidinone, tips were exposed to a 0.05 g/L solution of anti-human apoA1 in 0.1 M MES buffered saline, pH 4.7 by repetitively flowing (aspirating and dispensing 750 times) 50 μL volumes of antibody solution (150 μL/well) through the pipette tips. Tips were subsequently blocked with 1 M ethanolamine, pH 8.5 (400 μL/well; 50 x 150 μL aspirate and dispense steps) then exposed to 60 mMHCl 2*(400 μL/well; 50 x 150 μL aspirate and dispense steps) and equilibrated in HEPES buffered saline (HBS-N) 2*(400 μL/well; 50 x 150 μL aspirate and dispense steps). Antibody-linked pipette tips were stored in HBS-N at 4 °C until the day of use.

S1.3 Extraction of ApoA1

A batch of plasma samples were thawed, centrifuged at 10,600x g for 5 minutes and 50 μL of each sample was then aliquoted into a 96-well deep-well tray. The samples were diluted with 950 μL 10 mM HEPES-buffered saline (HBS-N). The samples were then exposed to the antibody-derivatized pipette frits by repeatedly aspirating and dispensing 75 μL of the samples 1000 times with the aid of a Beckman Multimek 96 channel automated pipetter. The protein loaded tips were washed by drawing and dispensing into waste (in 8 x 200 μL cycles each) HBS-N, H_2O, a 3:1 mixture of 2 M ammonium acetate:acetonitrile, and deionized H_2O. The tips were briefly air dried prior to elution of the protein. ApoA1 protein was eluted from the tips individually with 5.5 μL of formic acid/acetonitrile/deionized water (9/5/1 v/v/v) and deposited into a 96-

conical well polypropylene tray. The tip was then washed with an additional 5.5 μL of distilled water which was mixed with the other portion of the sample. The samples were then analyzed immediately (within 5 minutes of elution) by LC-ESI-TOF-MS. Since these particular samples were analyzed, we have a developed a patent-pending buffer formulation which allows for simultaneous elution of all tips in a batch and subsequent storage in a cooled, 96-well autosampler tray overnight. Specifically, without modification of the target protein(s) the buffer formulation prevents sample loss due to protein adsorption to vessel walls as well as artifactual oxidation of Met residues while samples await injection.

S1.4 LC-ESI-TOF-MS

Eight-microliter samples were injected without delay (within 5 minutes of elution to avoid protein formylation) by a Spark Holland Endurance autosampler in microliter pick-up mode and loaded by an Eksigent nanoLC*1D at 10 μL/min using 90% water / 10% acetonitrile with 0.1% formic acid onto a protein captrap configured for unidirectional flow on a 6-port diverter valve. The captrap was then washed for two minutes with this loading solvent. The flow rate over the protein captrap cartridge was then changed to 1 μL/min and a linear gradient of increasing acetonitrile from 10% to 90% was employed to elute the apoA1 protein into the mass spectrometer. The captrap eluate was directed to a Bruker MicrOTOF-Q (Q-TOF) mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the *m/z* range of 300 to 3000. ESI settings for the Agilent G1385A capillary microflow nebulizer ion source were as follows: End Plate Offset -500 V, Capillary -4500 V, Nebulizer nitrogen 2 Bar, Dry Gas nitrogen

3.0 L/min at 225 ° C. Data were acquired in profile mode at a digitizer sampling rate of 2 GHz. Spectra rate control was by summation at 1 Hz.

S1.5 Data Analysis

Approximately one minute of recorded spectra were averaged across the chromatographic peak apex of apoA1. The ESI charge-state envelope was deconvoluted with BrukerDataAnalysis v3.4 software to a mass range of 1000 Da on either side of any identified peak. Deconvoluted spectra were baseline subtracted and all peaks were integrated. Tabulated mass spectral peak areas were exported to a spreadsheet for further calculation and determination of the relative abundances (RAs) of each PTMs of apoA1, relative to all forms of apoA1.

S1.6 ApoA1 ESI-MSIA Analytical Pre-Validation

Prior to analyzing clinical samples, the precision, reproducibility and stability of apoA1 oxidation was confirmed: From a large volume of slightly oxidized stock plasma obtained from a single donor, three 1-mL aliquots of plasma were frozen and stored at -80 °C. On day one, the first 1-mL aliquot was thawed and divided into five subsamples that were then analyzed in a single batch using the apoA1 ESI-MSIA. This procedure was repeated on the following two days. The variation (%CV) of intra- and interday measurements of apoA1 oxidation was ~5% (**Supporting Information Table 1**). No biologically or statistically significant changes in the total amount of oxidation were noted between any of the days on which analysis took place. In practice we have found that oxidation does not occur during the protein isolation process as evidenced by the fact that some samples are completely free of oxides.

To further assess the oxidative stability of apoA1 under potential *ex vivo* conditions, a 2-mL aliquot of plasma from a healthy donor was allowed to sit on the bench top at room temperature. An aliquot of the sample was analyzed using the apoA1 ESI-MSIA assay at 0, 1, 2, 3, 4, 8, 12 and 16 days. Increased oxidation of apoA1 was seen after 3 days of incubation, and increased at a slow rate over time (**Supporting Information Figure 1**). Long term freezer storage (-80 °C) stability of apoA1 and its PTMs is currently under evaluation.

Finally, artifactual oxidation may also occur during the electrospray ionization process due to a phenomenon known as corona discharge which indirectly induces hydroxyl radical attack upon proteins as they are entering the gas phase [85]. Investigators should be aware that ion sources in which the high voltage is applied directly to the ESI needle is particularly susceptible to this spray-voltage dependent phenomenon. This problem can be circumvented, however, by employing an instrument (such as our Bruker MicroTOF-Q) with an ion source design in which the spray needle is held at ground and the entrance of the instrument itself is elevated to high negative voltage (in positive ion mode).

S2 Shotgun-based Multidimensional Protein Information Technology (MudPIT) Method

In our MudPIT experiments, peptide digests of the sample (1-2 micrograms) are injected (in technical replicates) onto a microbore HPLC system (Paradigm MS4, Michrom, Auburn, CA) with separate strong cation exchange (SCX) and reversed phase (RP) columns: a 250-µm I.D. capillary packed in-house with 7 cm of 5 µm Partisphere strong cation exchanger resin (Whatman, Clifton, NJ) and a 100 µm I.D. capillary packed in-house with 7 cm of 5 µm Vydac C18 reversed phase resin. The twelve steps that we employ in MudPIT analyses are described

in detail elsewhere [86]. Peptides eluting at 350 nL/min are sprayed into a ThermoFinnigan LCQ-Deca XP Plus ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) with a spray voltage of 1.6 kV. Spectra are scanned over the range 380-2000 mass units. Automated peak recognition, dynamic exclusion, and daughter ion scanning of the most abundant ions are performed using the Xcalibur v1.3 software as described elsewhere [87].

In our hands, all available data are used in designing transitions for MRM-based multiplexed analysis of HDL. Yet often a convenient starting point is to identify previously published transitions, if available—e.g., as described by Anderson and Hunter and by Kuzyk et al [70, 75].

S3 Overview of MRM-based Analyses

MRM in a triple quadrupole (QqQ) instrument offers the highest sensitivity for quantitation of analytes in complex mixtures. The non-scanning use of quadrupoles translates into an increased sensitivity up to two orders of magnitude compared with conventional full scan techniques [88]. In MRM, Q1 and Q3 act as filters to specifically select predefined *m/z* values corresponding to the parent peptide ions and daughter fragment ions, respectively. Q2 is used as a collision cell to produce the fragments that will be transmitted through Q3, and the collision energy is optimized for each parent/daughter pair. A transition is defined as the optimal *m/z* values for the parent/daughter as well as other optimal instrument-specific parameters that yield the maximum signal for each analyte. In a QTRAP 4000, these may include collision energy (CE), which is the applied voltage in q2 to fragment ions; declustering potential (DP), which is the voltage potential between the orifice plate and ground, optimized

to further strip off solvent molecules and reduce ion-ion aggregation; collision cell entrance potential (CEP); and collision cell exit potential (CXP).

In an MRM-based assay, a list of transitions is monitored as peptides are separated using LC—typically reverse phase with a C18 resin. As each transition is monitored in the mass spectrometer, a chromatogram for each transition is recorded. The area under the curve (AUC) value for each eluting peak corresponds directly to the abundance of the peptide analyte.

S3.1 Establishing MRM transitions

Although MRM assays can be designed using existing identifications of peptide fragments based on prior shotgun-style LC-MS/MS experiments, *in silico* resources, and spectral libraries, we did not find this approach to be reliable in predicting good MRM transitions for targeted proteins. We suggest generating new transitions from the protein of interest (digested recombinant or purified protein) unless these MRM transitions have been previously published. This type of analysis ensures that the sample preparation protocol is suitable for mass spectrometric analysis and that the subset of proteins and peptides selected for monitoring by MRM can be reproducibly quantified by the mass spectrometer.

Typically, to begin the surrogate peptide selection process one milligram of a recombinant protein (apoA1 for example) is reduced, alkylated and digested. Often a good starting approach to generating experimental data to aid in selection of surrogate peptides is to infuse the digested protein into the mass spectrometer without prior fractionation. The peaks representing the most abundant parent ions observed in single stage mass spectra are aligned with those of a theoretical protein digest. Peptides with matching *m/z* values are subjected to

MS/MS using collision energy and declustering potential ramps to generate optimized fragment ion spectra. Peptide identities are subsequently confirmed by matching them to theoretical fragmentation patterns generated, for example, using the UCSF Protein Prospector software. At this point the best SIS peptide candidates can be synthesized and subsequently tested in biological samples. But this quick leap to SIS synthesis is not always successful due to potential interferences in the initial MS scan created by the large number of peptides in the sample; indeed, the lack of fractionation can make peptide selection from single stage mass spectra of infused peptide digests rather difficult.

A more pragmatic approach is to confirm the surrogate peptide candidates identified during digest infusion by loading the sample onto an HPLC column in front of an *LTQ* (linear ion trap mass spectrometer equipped with an electrospray ionization source) or on an orbitrap (offers improved sensitivity and resolving power) and carrying out a shotgun-style LC-MS/MS experiment. This generates a list of abundant candidate peptides accompanied by experimental fragmentation data. This list is then aligned with the list of surrogate peptide candidates from the infusion experiment and the best proteotypic peptide candidates selected. SIS peptides are then synthesized and infused into the QTRAP in LC-MS/MS and LC-MRM modes for transition determination and optimization (see infusion section below). If desired, the digest can then be re-analyzed in LC-MRM mode (without full optimization of MS/MS parameters) to help confirm which peptides transitions are the top candidates. To optimize analytical sensitivity and specificity we recommend selecting at least three proteotypic surrogate peptides for each protein for which to synthesize SIS analogs. Upon acquisition, these pure SIS

peptides can be infused into the mass spectrometer to generate fully optimized MRM transitions.

In selecting peptides as surrogates of the target protein for MRM-based quantification, it is best to avoid peptides that contain amino acids or PTMs that are prone to chemical modification (such as Cys and Met) and/or rearrangements. Additionally, the peptides should be generated reproducibly, day after day under the specified proteolytic digest conditions. For more information on the process of selecting MRM transitions see the review published by Yocum et al in 2009 [76].

S3.2 Illustrative Experimental Details for Protein Digest or Peptide Infusions

We infuse digested protein solutions (or SIS peptides) by nanoelectrospray using a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a nanospray ionization source. A digest of 2 micrograms of protein diluted 10^2 to 10^4, or 1 microgram of peptide diluted by 10^3 suspended in 30% acetonitrile, 0.1% formic acid is infused at a flow rate of 300 nl/min using a Harvard PicoPlus 11 syringe pump (Harvard Apparatus, Holliston, MA). MS analysis is conducted in the positive ion mode with ion spray voltages in the 1800–2000-V range. The declustering potential is ramped (0–120 V in 2-V increments) during Q1 scans centered on 10-Da-wide mass ranges. MRM scans for optimization of MRM Q1/Q3 ion pairs is conducted with both Q1 and Q3 set to unit resolution (0.6–0.8-Da full width at half-height) while the collision energy is ramped (5–120 V in 2-V increments). Once the target peptide is selected with the ideal set of transitions, stable isotope peptides (SIS) are

synthesized and a second set of infusion MRMs on the SIS are done to confirm their transitions, collision energy and declustering potential.

S3.3 Illustrative Experimental Details for LC-MRM Analysis of Protein Digests

We employ an Eksigent NanoLC- 1D plus HPLC for the injection of desalted HDL digest samples (1 µl) onto reversed-phase capillary columns (75 µm X 15 cm) as previously described [75]. A flow rate of 300 nl/min solvent A (2% acetonitrile, 0.1% formic acid) is used for 6 min. Separations are performed using a flow rate of 300 nl/min with a 32-min linear gradient from 0 to 23% solvent B (98% acetonitrile, 0.1% formic acid) followed by a 9-min linear gradient from 23 to 43% solvent B. Our mass spectrometer for all MRM analyses is an Applied Biosystems/MDS Sciex 4000 QTRAP with a nanoelectrospray ionization source controlled by Analyst 1.5 software**.**