Supplemental Material for Hoofnagle, et al., 2011

METHODS

Human and animal specimens. The use of human specimens was approved by the Human Subjects Division at the University of Washington (UW). The collection and use of animal specimens was approved by the UW Institutional Animal Care and Use Committee.

HDL purification and proteolytic digestion. HDL was isolated from 335 µL EDTA-anticoagulated plasma using sequential ultracentrifugation (d=1.063-1.21 mg/mL), as previously described.[1-3] Mouse HDL was isolated using the same protocol. Five samples were chosen at random and pooled for MRM method development and for assay validation. HDL was frozen and stored at -80°C and thawed immediately prior to analysis.
For digestion HDL (10 µg protein) was solubilized with 0.2% RapiGest (Waters, Milford, MA) in 100 mM ammonium bicarbonate, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (1:20, w/w HDL protein; Promega, Madison, WI) for 3 h at 37°C. A second aliquot of trypsin (1:20, w/w HDL protein) was added and samples were incubated overnight at 37°C. After acidic hydrolysis of RapiGest with 0.5% trifluoroacetic acid, samples were dried, and stored at −20°C until analysis. Prior to analysis, samples were reconstituted in 5% acetonitrile, 0.1% formic acid.

Shotgun LC-MS/MS. Peptides from tryptic digests of human HDL (2 µg protein) were analyzed using an LTQ-Orbitrap XL (Thermo Fisher, San Jose, CA) equipped with a NanoAquity HPLC (Waters). A C18 trap column packed in-house (Magic AQ C18 200 Å, 5 µm, 0.1 x 20 mm, Michrom Bioresources, Auburn, CA) was used to desalt peptides for 15 min with water/0.1% formic acid (4 µL/min). Peptides were eluted onto an analytical column packed in-house (Magic AQ C18 100 Å, 5 µm, 0.1 x 200 mm, Michrom Bioresources), and separated at a flow rate of 0.4 µL/min over 180 min, using a linear gradient of 5% to 35% acetonitrile/0.1% formic acid in water/0.1% formic acid on a NanoAquity HPLC (Waters). Survey mass spectra were obtained at a resolution of 60,000 and MS/MS spectra were obtained in a data-dependent manner for the 8 most abundant ions in the survey scan. An exclusion window of 45 s was used after 2 repeated acquisitions of the same precursor ion.

Shotgun protein identification. MS/MS spectra were matched against the human International Protein Index (IPI) database[4] v3.72 using SEQUEST (v2.7) with fixed Cys carbamidomethylation and variable Met oxidation modifications. The mass tolerance for precursor ions was 50 ppm (LTQ-Orbitrap data) and SEQUEST default tolerance was accepted for product ions. No enzyme specificity was set for the search, but the results were filtered post-search to eliminate non-tryptic peptides from the analysis. SEQUEST results were further validated with PeptideProphet and ProteinProphet,[5,6] using an adjusted probability of ≥0.90 for peptides and ≥0.95 for proteins.

Shotgun protein quantification. Protein abundance was estimated using spectral counts—the total number of MS/MS spectra detected for a protein.[7-9] Each charge state of a peptide was considered a unique identification. Proteins considered for analysis were detected in ≥4 analyses with ≥2 unique identifications. When MS/MS spectra could not differentiate between protein isoforms, the isoform with the largest number of unique peptides was used for further analysis. For the 6 selected proteins quantification was also accomplished using extracted ion chromatograms. Chromatograms for peptides were extracted for the predominant charged states using LC Quan software v2.0 (Thermo Fisher) with precursor (m/z) mass window of ±0.01 Da and peak areas were integrated using the Genesis algorithm in LC Quan. The acquisition parameters detailed above provided at least 10 survey scans for a typical chromatographic peak to give at least 10 points across a peak for XIC quantitation.

Synthesis of stable isotope-labeled apoA-I protein. ^{15}N-labeled apoA-I was expressed in E. coli through a pET-20b bacterial expression vector encoding the human apoA-I sequence and an N-terminal His-tag.[10] Bacteria were grown in minimal medium with ^{15}N-ammonium chloride as the only source of nitrogen and the His-tagged protein was purified by Ni-affinity chromatography as described.[10] The His-tag does not interfere with the structure and function of the recombinant protein and was not removed.[11]

Selection of the peptides and transitions for MRM analysis. Two peptides for each protein were chosen for MRM analysis based on spectral count in the shotgun experiment and observed frequency in the PeptideAtlas mass spectral database.[12] Peptides containing methionine residues or known glycosylation sites were excluded from consideration. Predicted proteotypic properties were calculated using two prediction algorithms (STEPP[13,14] or PeptideSieve[13]) to ascertain that selected peptides had favorable proteotypic properties. All peptides selected scored high in both or at least one program confirming the empirical selection. The transitions monitored for each peptide were selected based on signal intensity from the shotgun experiment. Stable isotope-labeled peptides for two peptides for each protein were synthesized with ^{15}N-labeled and ^{13}C-labeled arginine (R*, $^{13}C_6H_{14}O_2{}^{15}N_4$) or lysine (K*, $^{13}C_6H_{14}O_2{}^{15}N_2$) at the C-terminus with purity better than 90%.

Multiple-reaction monitoring analysis. To quantify proteins in HDL using LC-MRM/MS, isotope-labeled apoA-I was added to HDL (1 µg ^{15}N-apoA-I per 10 µg HDL protein) prior to digestion (IS_{prot}) and isotope-labeled peptides (final concentration 50 nM) were added after digestion (IS_{pep}). Tryptic digests of HDL were chromatographed using a Tempo 1D Plus HPLC (AB Sciex, Foster City, CA) with a C18 trapping column (Acclaim Pepmap 100 Å, 5 µm, 5 x 1.0 mm, Dionex, Sunnyvale, CA)(trapping flow rate XX ul/min), a capillary C18AQ analytical column (Magic 200 Å, 5µm, 150 x 0.15 mm, Michrom), and an 8 min linear gradient of acetonitrile (5%-40%) in 0.2% formic acid in water at a flow rate of 1 µL/min. The Tempo 1D Plus HPLC was connected to a 4000 Q-TRAP mass spectrometer equipped with a MicroIonSpray II ion source (AB Sciex) and a silica emitter (Picotip FS360-20-10-C12, New Objective, Woburn, MA). Relevant instrument parameters were: spray tip voltage: 3,100 V, sheath gas parameter: 27, curtain gas parameter: 20. The instrument was operated in MRM mode with 10 ms dwell time and the peptides and internal standards were monitored using the transitions and collision energies listed in Supplementary Table 4. Peak areas were integrated using Analyst 1.4.2 (AB Sciex) with ≥12 points across each curve.

The transitions monitored for each peptide are listed in Supplementary Table 4. The abundance of each protein was calculated from MRM data as described follows: MRM peak area, the summed signal from both transitions monitored for each endogenous peptide; MRM-peptide ratio, the endogenous MRM peak area divided by the peak area of the respective synthesized internal standard peptide; MRM-protein ratio, the endogenous MRM peak area divided by the peak area of a peptide from the internal standard protein; normalized MRM-peptide and normalized MRM-protein ratio, the MRM-peptide ratio or the MRM-protein ratio for each peptide were normalized to the mean across the sample set and the two peptides were averaged. Peptides used for normalization are listed in Supplemental Table 3B,C.

Immunoassays. ApoA-I concentration was quantified using nephelometry (BN II, Siemens, Deerfield, IL). Prior to analysis, 5 µL HDL was diluted with 95 µL sample diluent and the measured concentration was normalized to the protein content in HDL. The method has good inter-day reproducibility (4.6% CV, N=90). Concentrations of ApoB, ApoC-II, ApoC-III, and ApoE were measured using sandwich immunoassays (MILLIPLEX MAP Human Apolipoprotein Panel 6, Millipore, Billerica, MA). The inter-day reproducibility of each assay is <22% CV, as reported by the manufacturer. ApoJ was quantified using a sandwich immunoassay (BioVendor, Candler, NC) with <8.5% CV, as reported by the manufacturer.

Precision and linearity experiments. To evaluate the repeatability of the LC-MRM/MS step, digested pooled HDL was analyzed 35 times over 3 d and 4 d later 24 times over 2 d (total of 9 d). The average %CV was calculated as the average %CV observed for each normalized MRM-protein ratio or normalized MRM-peptide ratio for the two experiments for each of the proteins. To evaluate the repeatability of the digestion step, pooled HDL was digested 5 times and analyzed twice on day one and twice on day nine. The average of the four injections was used as the observed concentration for each digestion and the %CV of the observed concentrations across the 5 digests was used as the %CV of the digestion. To estimate the overall variability of the digestion and LC-MRM/MS of the assay, we used the sum of squares approach as previously described:[15]

$$\%CV\ total = \sqrt{((\%CV\ LC\text{-}MRM/MS)^2 + (\%CV\ Digestion)^2)}$$

To establish linearity, human HDL was diluted into mouse HDL to maintain the total protein amount at 10 µg (range of human HDL, 25-100% human HDL), each sample was digested once and analyzed using LC-MRM/MS.

Statistical analysis. Distributions were tested for normality using the method of Shapiro-Wilk and the variables were log-transformed if skewed (apoB). Pearson correlation coefficients (r or r^2) were then calculated. All calculations were performed in the R statistical computing environment (http://www.r-project.org).

Supplemental Table 1. Proteins detected by shotgun proteomics.
Proteins selected for the study highlighted in bold

Protein	Gene	Description	Mean[a]	SD	CV %
IPI00021841	APOA1	**Apolipoprotein A-I**	656.0	93.2	14.2%
IPI00021854	APOA2	Apolipoprotein A-II	351.3	92.8	26.4%
IPI00022229	APOB	**Apolipoprotein B-100**	126.5	52.9	41.8%
IPI00021855	APOC1	Apolipoprotein C-I	77.5	16.8	21.7%
IPI00021857	APOC3	**Apolipoprotein C-III**	60.2	10.5	17.4%
IPI00022434	ALB	Albumin	52.6	13.3	25.4%
IPI00021842	APOE	**Apolipoprotein E**	45.9	10.0	21.9%
IPI00021856	APOC2	**Apolipoprotein C-II**	41.6	10.8	25.9%
IPI00218732	PON1	Serum paraoxonase/arylesterase 1	40.5	8.7	21.6%
IPI00006662	APOD	Apolipoprotein D	38.4	5.7	14.8%
IPI00019399	SAA4	Serum amyloid A4	23.2	6.9	29.9%
IPI00164623	C3	Complement C3	23.2	5.8	25.1%
IPI00186903	APOL1	Isoform 2 of Apolipoprotein L1	18.8	3.3	17.6%
IPI00030739	APOM	Apolipoprotein M	17.2	3.0	17.3%
IPI00479805	APOA4	Apolipoprotein A-IV	16.9	4.4	26.1%
TRYP_PIG	TRYP	Porcine trypsin	15.6	2.5	16.0%
IPI00299435	APOF	Apolipoprotein F	14.3	5.4	37.5%
IPI00291262	CLU	**Clusterin (apoJ)**	13.0	3.1	24.1%
IPI00006146	SAA1	Serum amyloid A2	12.4	7.0	56.6%
IPI00299778	PON3	Serum paraoxonase/lactonase 3	10.6	3.7	35.3%
IPI00553177	SERPINA1	Alpha-1-antitrypsin	10.5	4.8	46.2%
IPI00032258	C4A	Complement component C4A	9.2	4.7	51.1%
IPI00384280	PCYOX1	Prenylcysteine oxidase 1	8.9	3.1	34.5%
IPI00022731	APOC4	Apolipoprotein C-IV	7.9	2.3	29.8%
IPI00029168	LPA	Apolipoprotein(a)	7.8	13.9	177.0%
IPI00552578	SAA1	Serum amyloid A	7.1	4.5	63.3%
IPI00477597	HPR	Haptoglobin-related protein	5.5	2.3	41.3%
IPI00022432	TTR	Transthyretin	4.2	1.8	43.3%
IPI00021891	FGG	Fibrinogen gamma chain	3.4	2.2	65.0%
IPI00022331	LCAT	Phosphatidylcholine-sterol acyltransferase	3.0	0.9	30.3%
IPI00382606	FVII	Factor VII	3.0	1.8	61.0%
IPI00431645	HP	HP protein	2.7	1.7	63.3%
IPI00418163	C4B	Complement component C4B	2.6	1.3	48.3%
IPI00061977	IGHA1	IGHA1 protein	2.4	2.0	84.1%
IPI00036552	ANTXR2	Isoform 2 of Anthrax toxin receptor 2	2.2	1.5	65.1%
IPI00298971	VTN	Vitronectin	2.0	1.6	78.0%
IPI00298497	FGB	Fibrinogen beta chain	2.0	2.1	108.9%
IPI00022733	PLTP	Phospholipid transfer protein	1.9	1.6	82.2%
IPI00011588	PLA2G7	Platelet-activating factor acetylhydrolase	1.8	1.5	81.1%

[a] Mean spectral counts across the clinical study population of 30 subjects.

Supplemental Table 2. Proteotypic analysis of peptides. Chosen peptides were analyzed with two different algorithms for proteotypic peptide prediction. Proteotypic peptides are those peptides that are unique in the human genome and ionize well in mass spectrometric analyses.

		STEPP		PeptideSieve	
Protein	**Peptide**	**Score**	**Rank**	**Score**	**Rank**
ApoA-I	DLATVYVDVLK	0.6246	3	0.8735	7
ApoA-I	VQPYLDDFQK	0.5782	6	[a]	-
ApoB	NPNGYSFSIPVK	0.5945	71	0.8124	[b]
ApoB	SVSLPSLDPASAK	0.7361	30	0.8330	[b]
ApoB	VSALLTPAEQTGTWK	0.5947	70	0.8237	[b]
ApoC-II	ESLSSYWESAK	0.2523	4	0.8003	4
ApoC-II	TYLPAVDEK	0.6940	1	0.8279	2
ApoC-III	DALSSVQESQVAQQAR	0.4256	3	0.8767	4
ApoC-III	GWVTDGFSSLK	0.3997	5	0.8205	5
ApoE	LAVYQAGAR	0.2407	13	0.9346	2
ApoE	SELEEQLTPVAEETR	0.7405	3	0.8615	3
ApoE	VEQAVETEPEPELR	0.7833	2	0.8479	4
ApoJ	LFDSDPITVTVPVEVSR	0.8999	1	[a]	[a]
ApoJ	ASSIIDELFQDR	0.5886	5	0.9950	1

[a] not predicted
[b] rank not available

Supplemental Table 3. Tryptic peptides used in the study and associated fragment ions.

A. Endogenous peptides

Protein	Peptide sequence	IS_{prot}[a]	Mass	Q1[b]	CE[c]	Q3-1[b]	ion	Q3-2[b]	ion	XIC
ApoA-I	DLATVYVDVLK	N1	1234.68	618.34	20, 20	736.42	y_6	936.54	y_8	Contaminated[d]
ApoA-I	VQPYLDDFQK	N2	1251.61	626.81	40, 20	422.24	y_3	1025.49	y_8	626.81
ApoA-I	DYVSQFEGSALGK[e]	-	1399.66	-	-	-	-	-	-	700.84
ApoB	SVSLPSLDPASAK	N2	1270.68	636.34	29, 17	473.27	y_5	885.47	y_9	636.35
ApoB	VSALLTPAEQTGTWK	N2	1600.85	801.43	43, 43	1017.50	y_9	1118.55	y_{10}	801.43
ApoC-II	ESLSSYWESAK	N2	1285.58	643.79	30, 30	620.30	y_5	870.40	y_7	643.80
ApoC-II	TYLPAVDEK	N2	1034.53	518.27	24, 25	658.34	y_6	771.42	y_7	518.27
ApoC-III	DALSSVQESQVAQQAR	N2	1715.84	858.92	42, 42	502.27	y_4	672.38	y_6	572.96
ApoC-III	GWVTDGFSSLK	N2	1195.59	598.80	25, 26	854.43	y_8	953.49	y_9	598.80
ApoE	LAVYQAGAR[f]	N2	947.52	474.76	20, 20	665.34	y_6	764.41	y_7	Contaminated[d]
ApoE	SELEEQLTPVAEETR	N2	1729.84	865.92	40, 40	801.41	y_7	902.46	y_8	865.90
ApoE	AATVGSLAGQPLQER[e]	-	1496.79	-	-	-	-	-	-	749.40
ApoJ	LFDSDPITVTVPVEVSR	N2	1872.98	937.49	40, 40	578.25	b_5	686.38	y_6	937.50
ApoJ	ASSIIDELFQDR	N2	1392.69	697.35	40, 40	1035.50	y_8	922.43	y_7	697.35

[a] N1, N2 indicates ^{15}N-apoA-I peptide used as an internal standard in IS_{prot} approach (see Supplemental Table 3C).

[b] Two transitions were monitored for each peptide (m/z for precursor notes as **Q1**). The two fragment ions monitored for each peptide are denoted **Q3-1** and **Q3-2**.

[c] Collision energy is listed for each peptide (shown as: **CE** for Q3-1, **CE** for Q3-2).

[d] One peptide from apoA-I and one peptide from apoE that were used in the MRM analysis had significant interference in the XIC chromatogram. As a result, we chose a different peptide for the XIC analysis.

[e] Peptide used for extracted ion chromatogram (**XIC**) only.

[f] Peptide LAVYQAGAR is polymorphic in humans. In three subjects with the R>C mutation, relative protein concentrations were quantified using only peptide SELEEQLTPVAEETR..

Supplemental Table 3. Tryptic peptides used in the study. (continued)

B. Stable isotope-labeled peptides

Protein	Peptide sequence[a]	Mass	Q1[b] (IS_{pep})	Q3-1[b] (IS_{pep})	ion	Q3-2[b] (IS_{pep})	ion
ApoA-I	DLATVYVDVLK	1242.68	622.35	744.42	y_6	944.54	y_8
ApoA-I	VQPYLDDFQK	1259.61	630.81	430.24	y_3	1033.49	y_8
ApoB	SVSLPSLDPASAK	1278.68	640.35	481.27	y_5	893.47	y_9
ApoB	VSALLTPAEQTGTWK	1608.85	805.43	1025.50	y_9	1126.55	y_{10}
ApoC-II	ESLSSYWESAK	1293.58	647.80	628.30	y_5	878.40	y_7
ApoC-II	TYLPAVDEK	1042.53	522.27	666.34	y_6	779.42	y_7
ApoC-III	DALSSVQESQVAQQAR	1725.84	576.29	512.27	y_4	682.38	y_6
ApoC-III	GWVTDGFSSLK	1203.59	602.80	862.43	y_8	961.49	y_9
ApoE	LAVYQAGAR	957.52	479.77	675.34	y_6	774.41	y_7
ApoE	SELEEQLTPVAEETR	1739.84	870.93	811.41	y_7	912.46	y_8
ApoJ	LFDSDPITVTVPVEVSR	1882.98	942.50	578.25	b_5	696.38	y_6
ApoJ	ASSIIDELFQDR	1402.69	702.35	1045.50	y_8	932.43	y_7

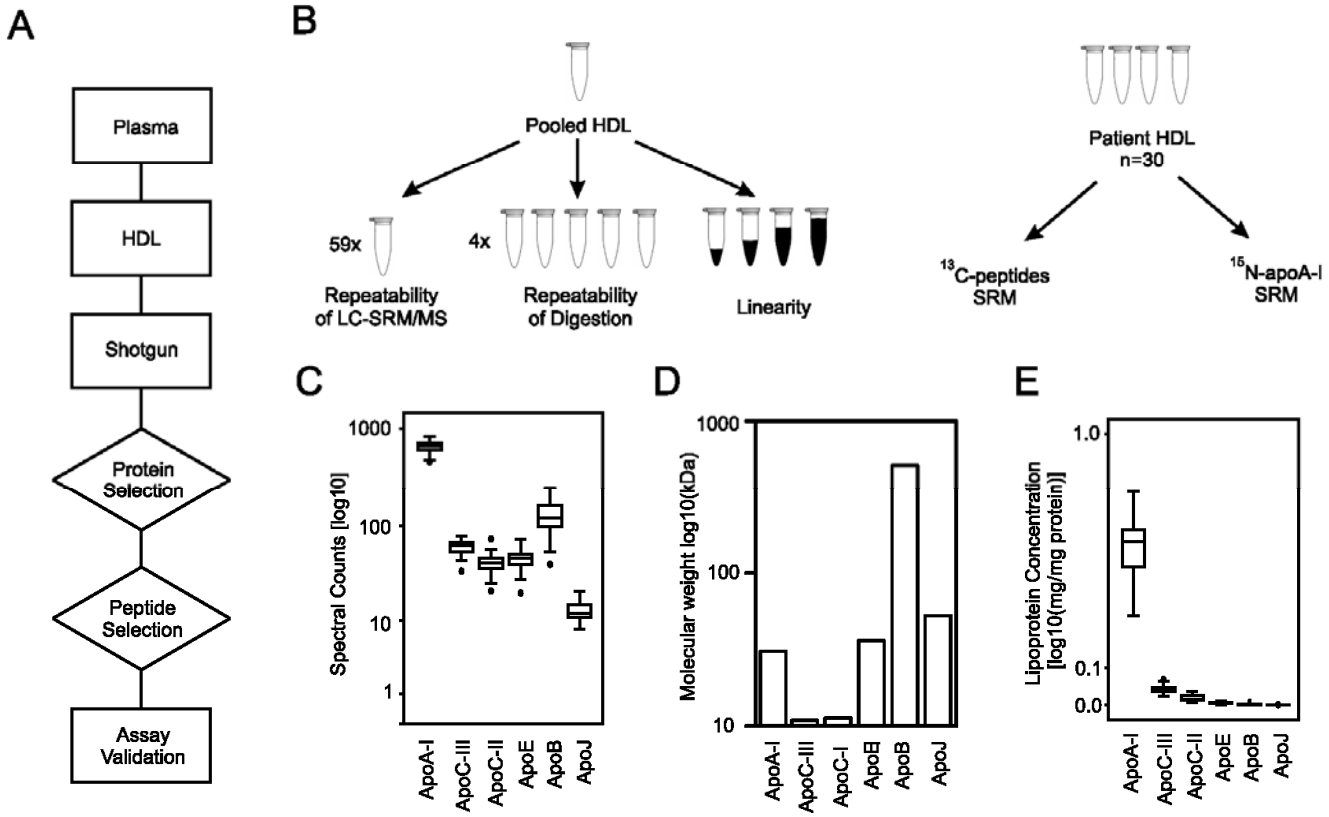
[a] Peptides were synthesized with isotope-labeled arginine or lysine at the carboxyl terminus.
[b] Two transitions were monitored for each isotope-labeled peptide (m/z for precursor notes as **Q1**). The two fragment ions monitored for each peptide are denoted **Q3-1** and **Q3-2**.

C. ^{15}N-labeled protein derived peptides (IS_{prot})

Protein	Peptide sequence	Peptide Code	Mass	Q1[a] (IS_{prot})	CE[b]	Q3-1[a] (IS_{prot})	ion	Q3-2[a] (IS_{prot})
ApoA-I	DLATVYVDVLK	N1	1246.68	624.33	20, 20	1017.55	y_9	945.51
ApoA-I	VQPYLDDFQK	N2	1264.61	633.29	40, 20	231.13	b_2	1035.46

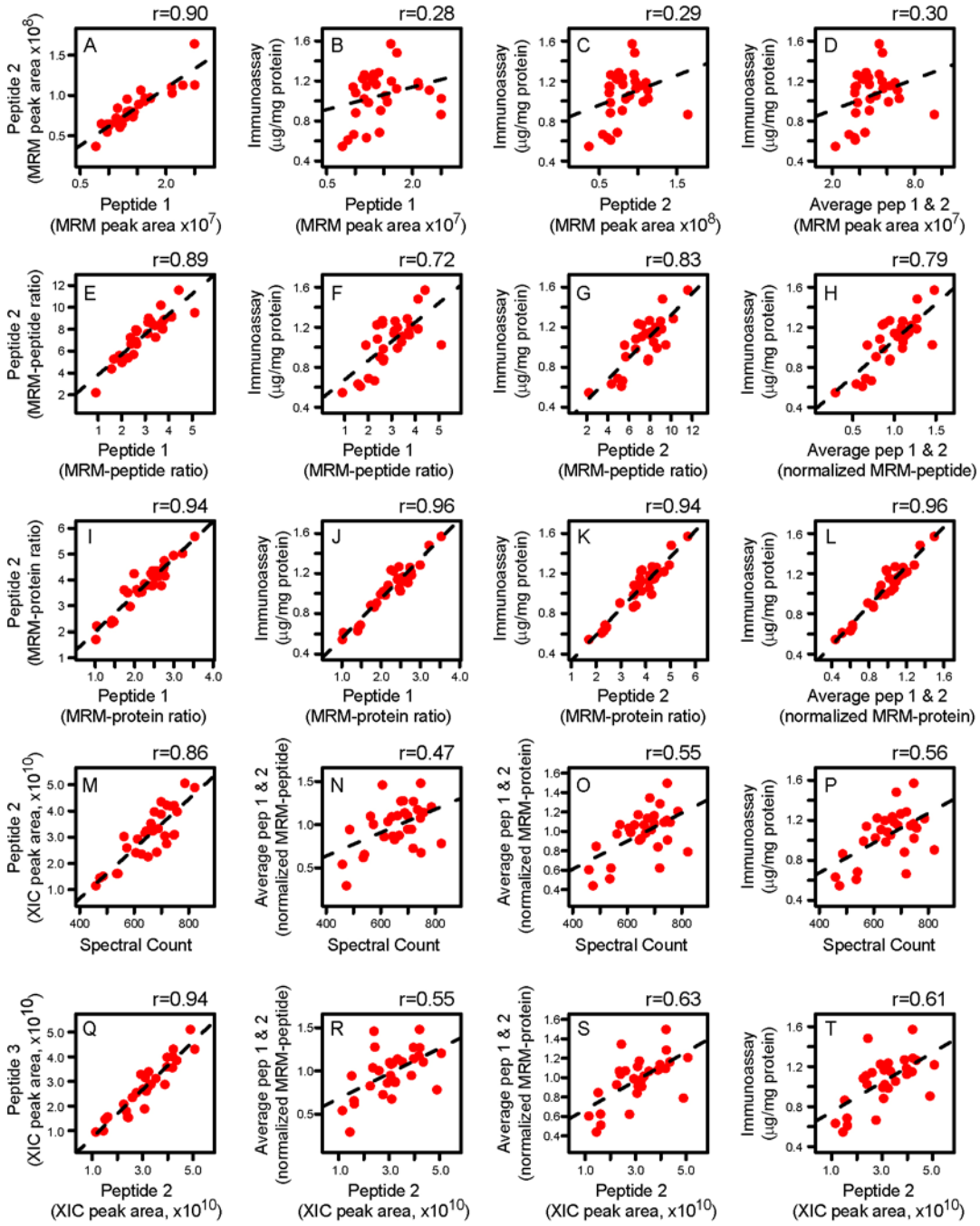
[a] Two transitions were monitored for each isotope-labeled peptide (m/z for precursor notes as **Q1**). The two fragment ions monitored for each peptide are denoted **Q3-1** and **Q3-2**.
[b] Collision energy for each peptide (shown as: **CE** for Q3-1, **CE** for Q3-2).

Supplemental Figure 1. Study outline and protein characteristics. (**A**) *Method development.* HDL was isolated from the plasma of 30 apparently healthy subjects and was characterized by shotgun proteomics analysis. A set of six HDL proteins were selected that: 1) had a range of concentrations across the study population that exceeded 50% of the population mean concentration, 2) had available immunoassays, and 3) together had relative abundances that spanned at least 3 orders of magnitude. (**B**) *Method validation.* After establishing the assay configuration, repeatability of the LC-MRM/MS step was assessed by injecting digested pooled HDL 35 times over 3 d and 24 times over 2 d (total 9 days). Repeatability of the overall assay was assessed by injecting 5 replicate digests of the pooled HDL twice each on day 1 and day 9. Linearity was assessed by serial dilution of human HDL into mouse HDL. (**C**) *Protein characteristics.* The six proteins selected for the study had concentrations between 0.32 and 740 µg/mg HDL protein, were detected in the shotgun experiment with an average of 13-656 spectral counts, and had molecular weights between 11 and 550 kDa.

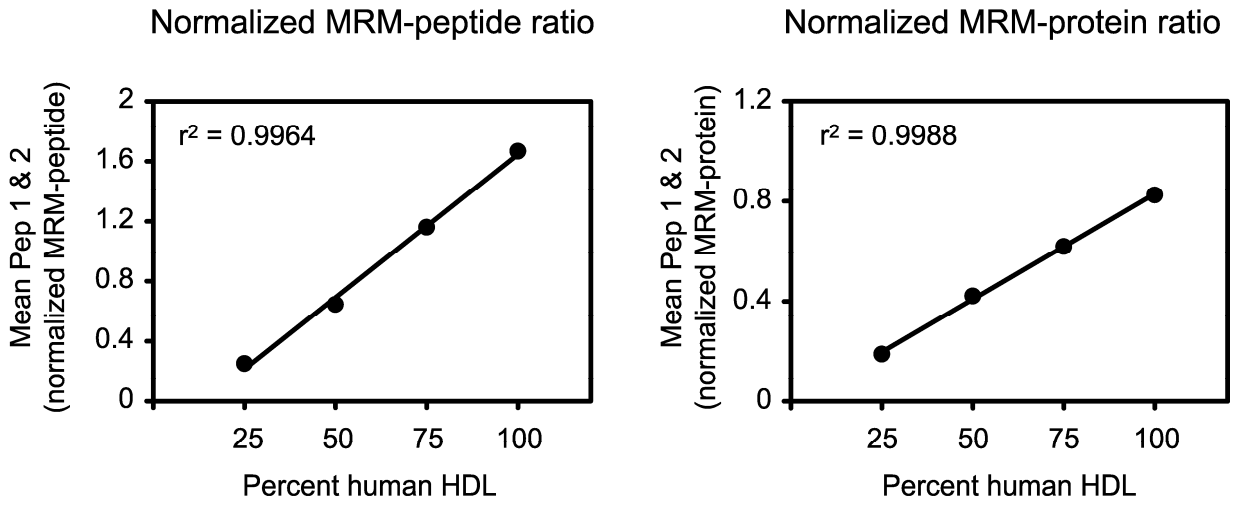


Supplemental Figure 2. Quantification of apoA-I in HDL. The chromatographic peak areas for both endogenous apoA-I peptides (VQPYLDDFQK, DYVSQFEGSALGK) were determined *(MRM peak area)*. Each endogenous peak area was divided by the peak area of the respective spiked internal standard peptide to calculate the *MRM-peptide ratio* or by the peak area of the peptide from the spiked internal standard protein to calculate the *MRM-protein ratio*. The *MRM-peptide ratio* and the *MRM-protein ratio* of each peptide were then normalized to the mean value across the whole sample set and averaged over the two peptides to calculate *normalized MRM-peptide ratio* and *normalized MRM-protein ratio* for each protein, respectively. Peak areas from extracted ion chromatograms for the precursor ions of two apoA-I peptides were determined from the high resolution MS1 scan in the shotgun experiment *(XIC)*. **(A-D)** *MRM-peak area* of each peptide was compared with each other and with the apoA-I nephelometric immunoassay (individually and as the *mean MRM peak area* of the two peptides). **(E-H)** *MRM-peptide ratio* for each peptide was compared as for *MRM-peak area*. **(I-L)** *MRM-protein ratio* for each peptide was compared as for *MRM-peak area*. **(M-P)** The *spectral count* for apoA-I was compared to the *XIC* peak area (acquired at the same time as the spectral count), *normalized MRM-peptide ratio*, *normalized MRM-protein ratio*, and the immunoassay. **(Q-T)** The *XIC* for two peptides were compared to each other and to the *normalized MRM-peptide ratio*, *normalized MRM-protein ratio*, and immunoassay.

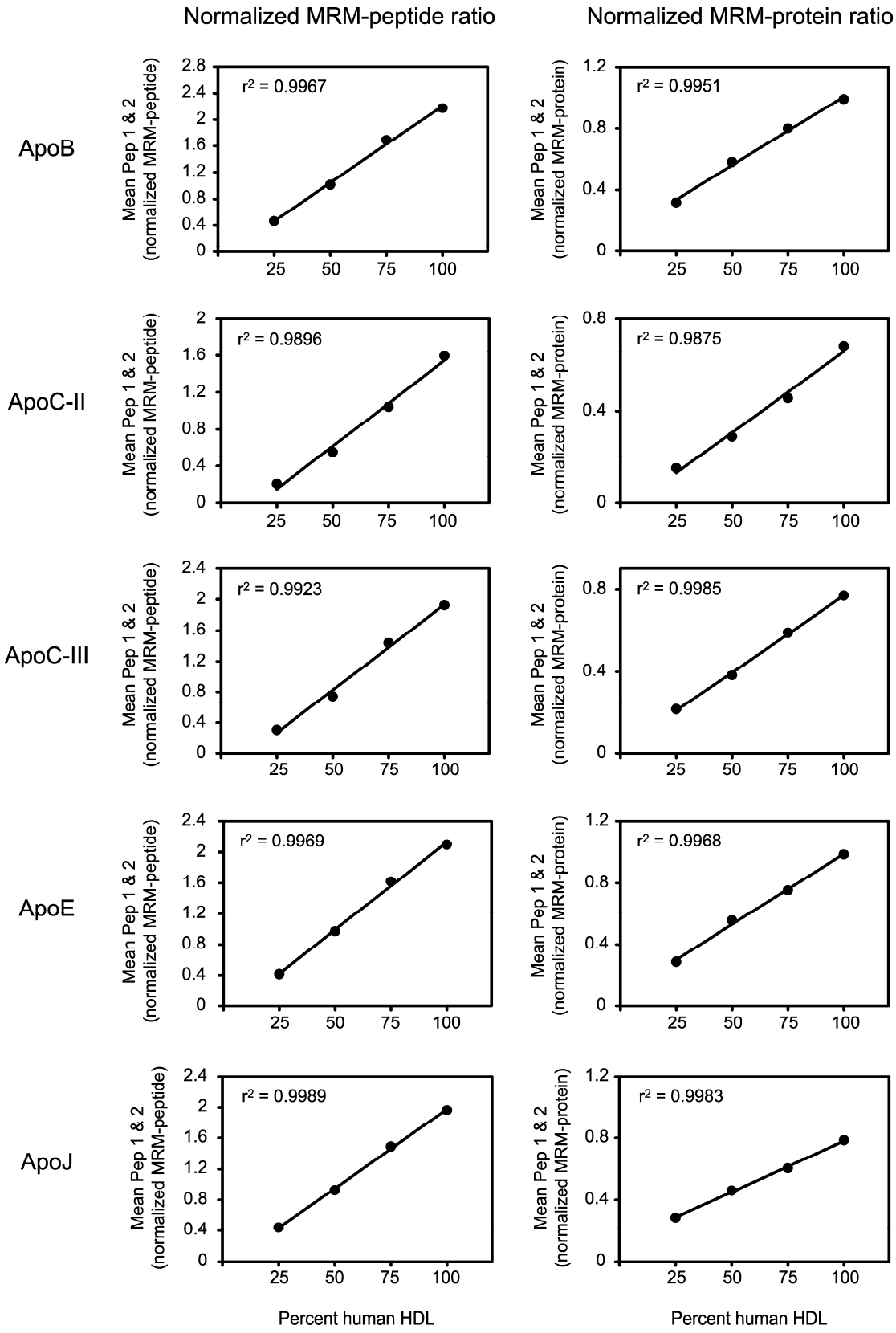
Supplemental Figure 2. Quantification of apoA-I in HDL.



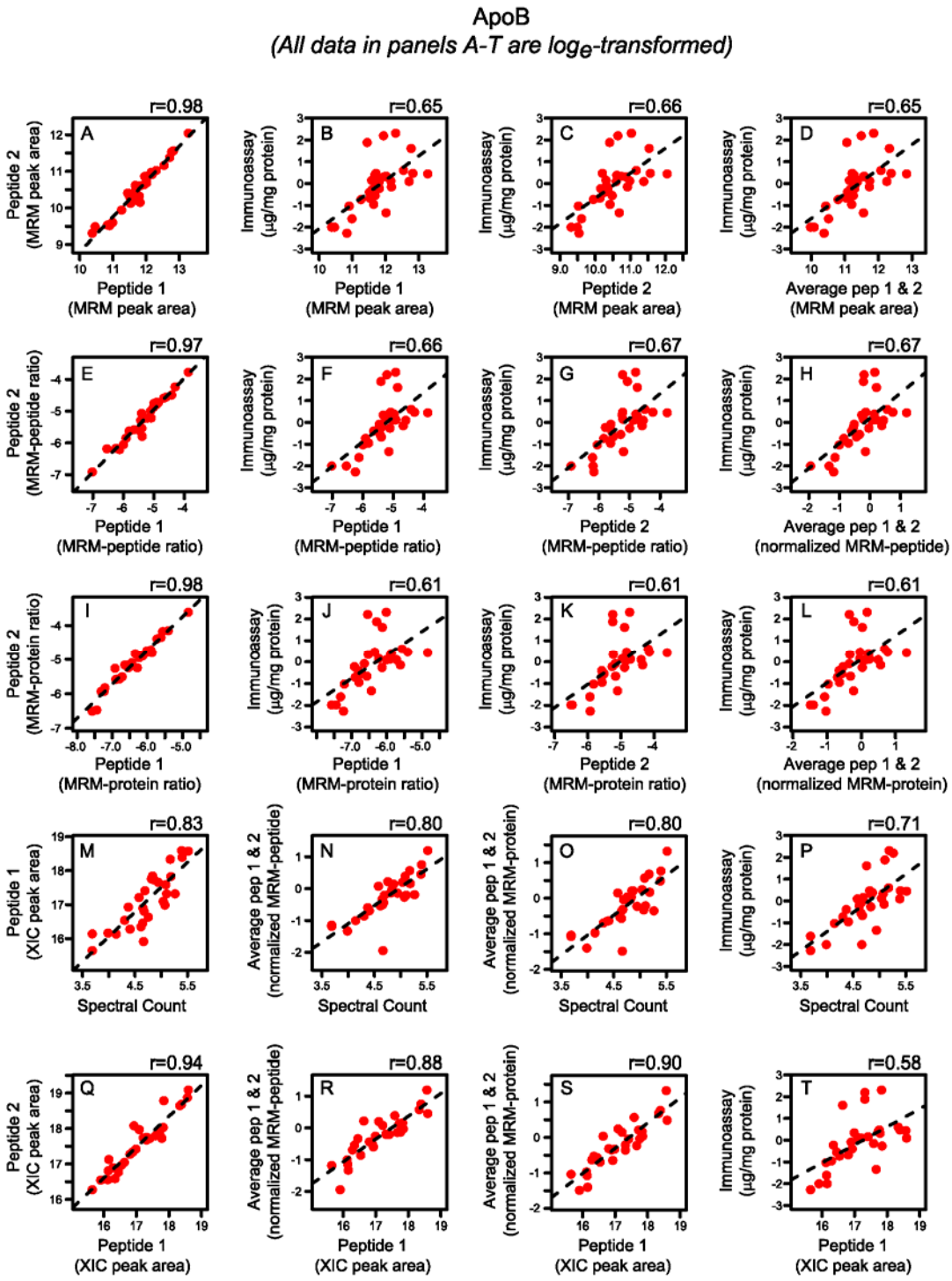
Supplemental Figure 3. Linearity of MRM-MS assays for apoA-I



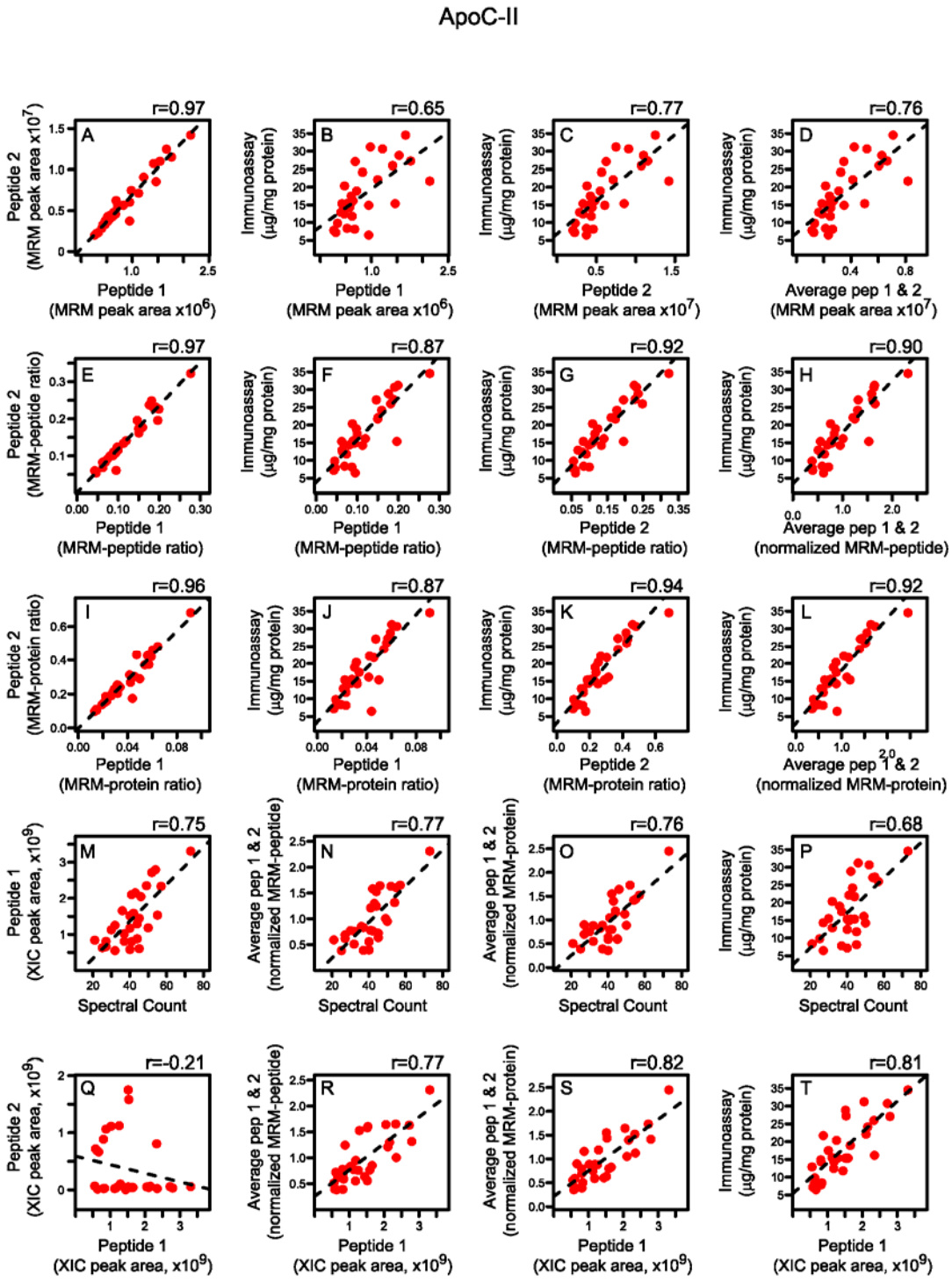
Supplemental Figure 4. Linearity of MRM-MS assays for 6 other proteins in HDL



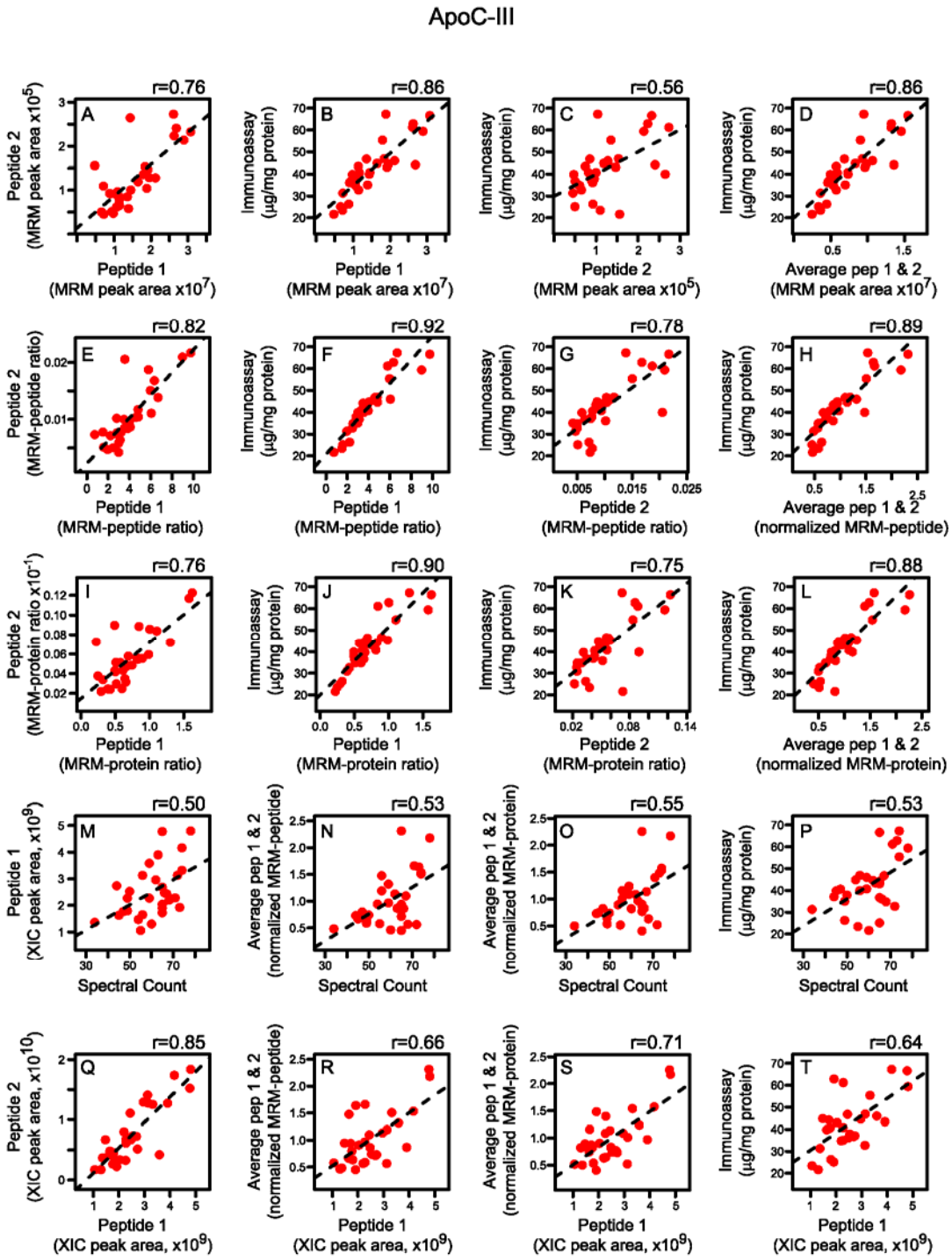
Supplemental Figure 5. Quantification of ApoB in HDL. Data are presented as in Supplemental Figure 2.



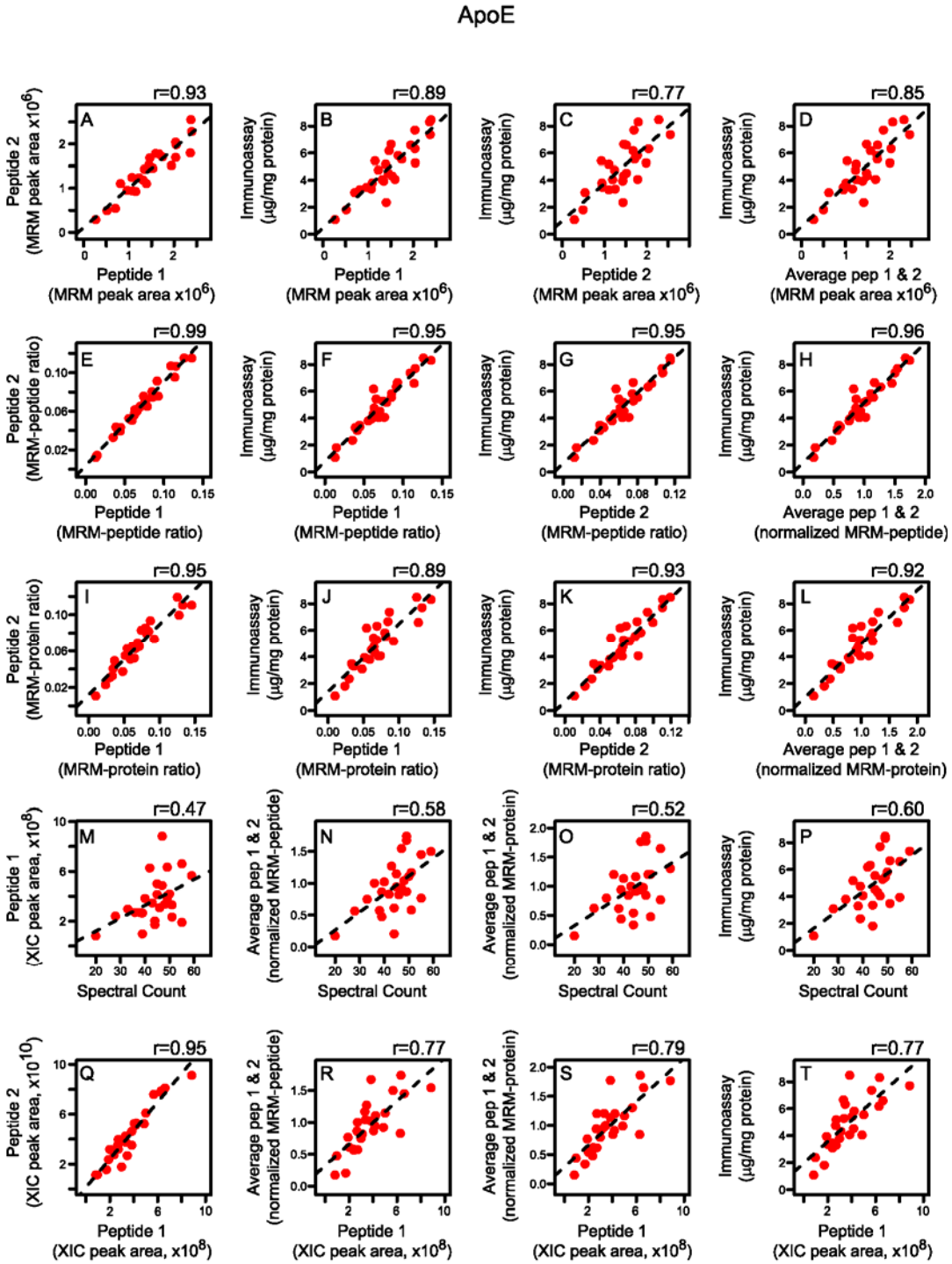
Supplemental Figure 6. Quantification of apoC-II in HDL. Data are presented as in Supplemental Figure 2.



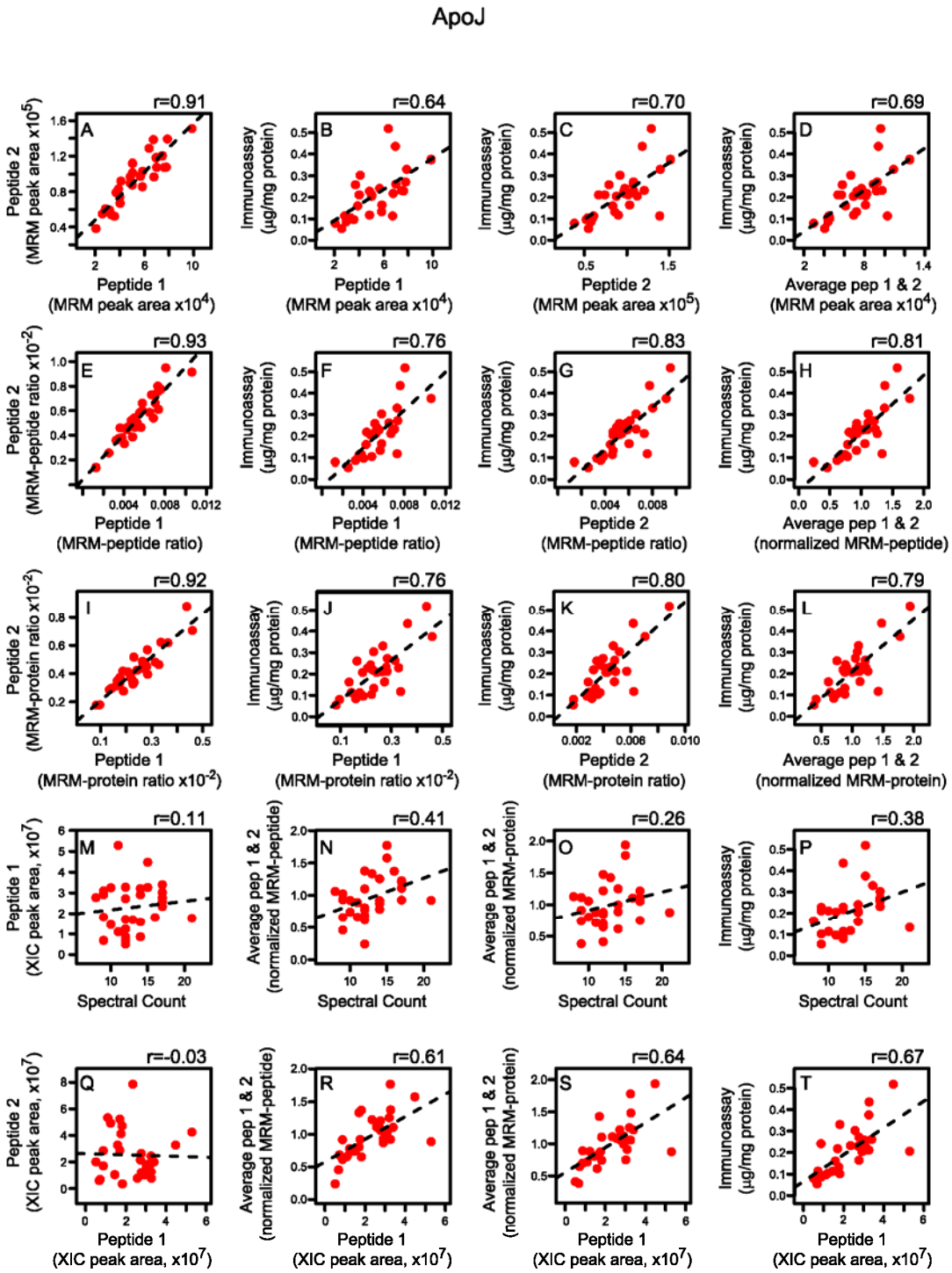
Supplemental Figure 7. Quantification of apoC-III in HDL. Data are presented as in Supplemental Figure 2.



Supplemental Figure 8. Quantification of apoE in HDL. Data are presented as in Supplemental Figure 2.



Supplemental Figure 9. Quantification of ApoJ in HDL. Data are presented as in Supplemental Figure 2.



SUPPLEMENTAL REFERENCES

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