Supplementary Figures

Supplementary Fig. 1. Functional analysis of 15 verified proteins from preliminary MRM analysis

The 15 verified proteins from the preliminary MRM analysis were classified under the ″biological process″ and ″molecular function″ categories using the PANTHER classification program (A). The largest components in ″biological process″ were ″immunity and defense″ (33%) and ″molecular function″ (20%), respectively. To investigate the signaling pathway related to the 15 verified proteins, we conducted signaling pathway analysis. Consequently, 2 signaling pathways (actin cytoskeleton and complement cascade pathway) were selected from the KEGG database (B and C).

Supplementary Fig. 2. Correlation of peak area values between two peptides per protein

The peak area values between two peptides per protein were compared in SQ-MRM experiments. The peak similarities of two peptides were analyzed by Pearson′s correlation analysis. The patterns of peak intensity were highly correlated between two peptides in each protein showing the median value of 0.927.

Supplementary Fig. 3. Confirmation of synthesized heavy peptide using the MILDI-TOF

To verify the 15 selected candidates, we first synthesized SIS peptides (>95% purity) for each protein and verified the purity by MALDI TOF analysis. All the SIS heavy peptides had the same theoretical and experimental molecular weight values.

Supplementary Fig. 4. Generation of calibration curve for 15 proteins

To generate a calibration curve for the 15 proteins, heavy peptides were serially diluted (12 concentration points: 0. 1−200 fmol) with the light peptide as an internal standard (digested pooled plasma protein: 1 μg) added into each serially diluted samples. Each experiment was repeated in triplicate to generate the calibration curve. The area ratio of heavy peptide (12 concentration points: 0.1−200 fmol) to light peptide (internal standard: plasma l μg) was determined from the extracted ion chromatogram (XIC) of each selected transition.

Supplementary Fig. 5. Evaluation of retention time stability in No DR, Mi NPDR, and Mo NPDR groups

The CV% of retention time (RT) for 15 peptides are observed in NO DR (N: 20), Mi NPDR (N: 20), and Mo NPDR groups (N: 20). The CV% for RT in three groups is shown in a range of 0.87-8.35, 0.23-4.81, and 0.11-4.92 min, respectively. The averaged RT for 15 peptides (D) represent in a range of 12.6-46.4 min. The peptide of ITIH2_VQSTITSR_y6++ is a first eluted peptide in 15 peptides (12.6 min), while the peptide of APLP2_WEPDPTGTK_y7++ is a last eluted peptide (46.4 min). All images were extracted by Skyline program.

Supplementary Fig. 6. Selection of multi-marker panel by statistical analysis

Multicolinearity among the 11 candidates was investigated using a linear regression analysis (A). To build multi-marker panel, stepwise MANOVA was employed to determine the suitability of candidates for inclusion in a multi-marker panel (B). From the stepwise MANOVA, 4 verified markers (ITIH2, APOA4, C7, and CLU) were selected and used to construct a multi-marker panel. Statistical validation was then performed using LOOCV.

Supplementary Fig. 7. Comparison of mean concentration in No DR, Mi NPDR, and Mo NPDR groups versus normal plasma concentration

To assess the measured concentration from SID-MRM analysis in No DR (n=20), Mi NPDR (n=20), and Mo NPDR (n=20) groups, the each mean concentration level was compared to normal plasma concentration reported in the literatures. 15 proteins were ordered from highest-to-lowest abundance with the LLOQ of each corresponding assay.

Supplementary Table Legends

Supplementary Table 1. Characteristics of clinical plasma samples in the No DR, Mi NPDR, and Mo NPDR groups

Supplementary Table 2. Statistics for clinical plasma sample in the No DR, Mi NPDR, and Mo NPDR groups

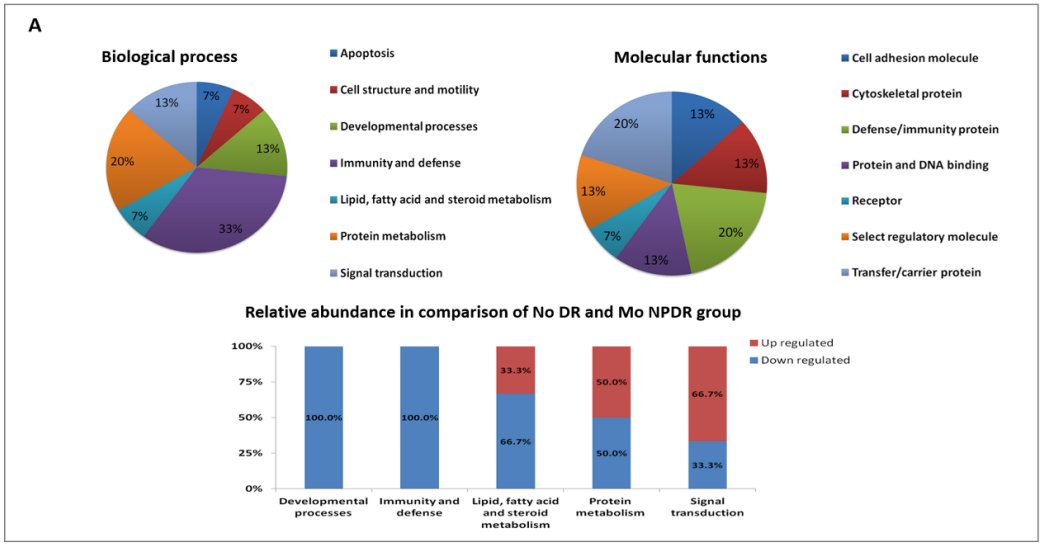
Supplementary Table 3. Identified human vitreous proteome in MH, NPDR, and PDR groups

Supplementary Table 4. Differentially expressed proteins in human vitreous

Supplementary Table 5. Data-mining for MRM target selection in human vitreous

Supplementary Table 6. Selection of reliable transitions for MRM analysis

Supplementary Fig. 1


A
Biological process
Apoptosis
Cell structure and motility
Developmental processes
Immunity and defense
Lipid, fatty acid and steroid metabolism
Protein metabolism
Signal transduction
7%
7%
13%
33%
7%
20%
13%
Molecular functions
Cell adhesion molecule
Cytoskeletal protein
Defense/immunity protein
Protein and DNA binding
Receptor
Select regulatory molecule
Transfer/carrier protein
13%
13%
20%
13%
7%
13%
20%
Relative abundance in comparison of No DR and Mo NPDR group
Up regulated
Down regulated
100%
75%
50%
25%
0%
100.0%
100.0%
33.3%
66.7%
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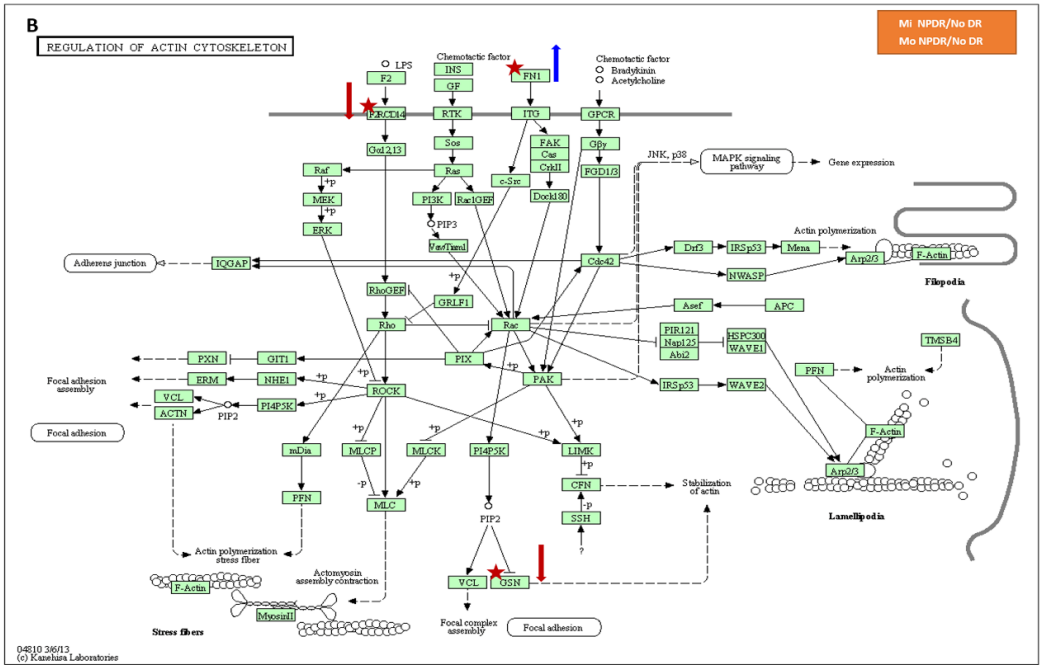
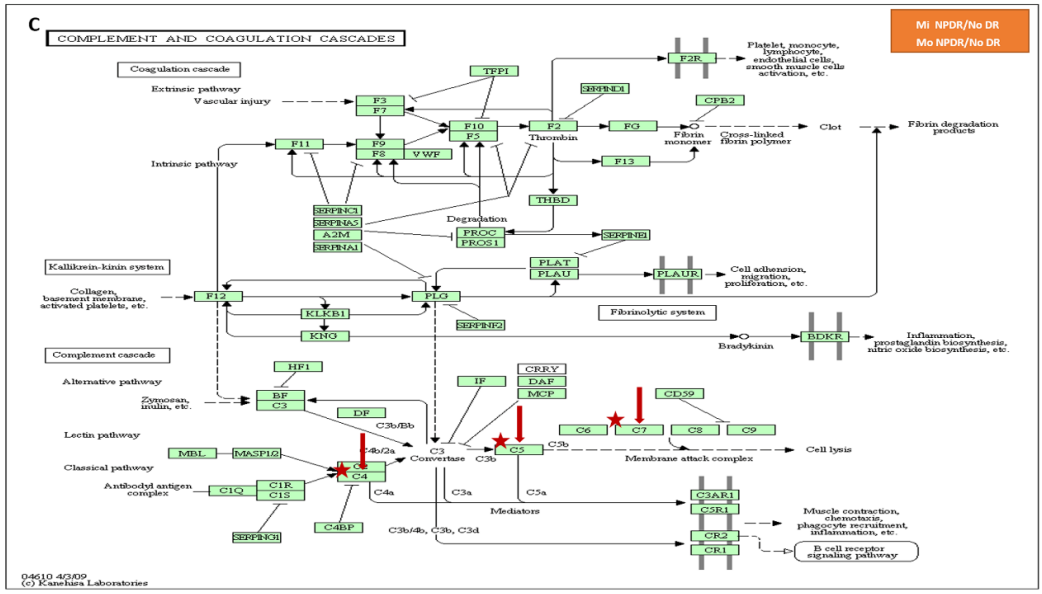
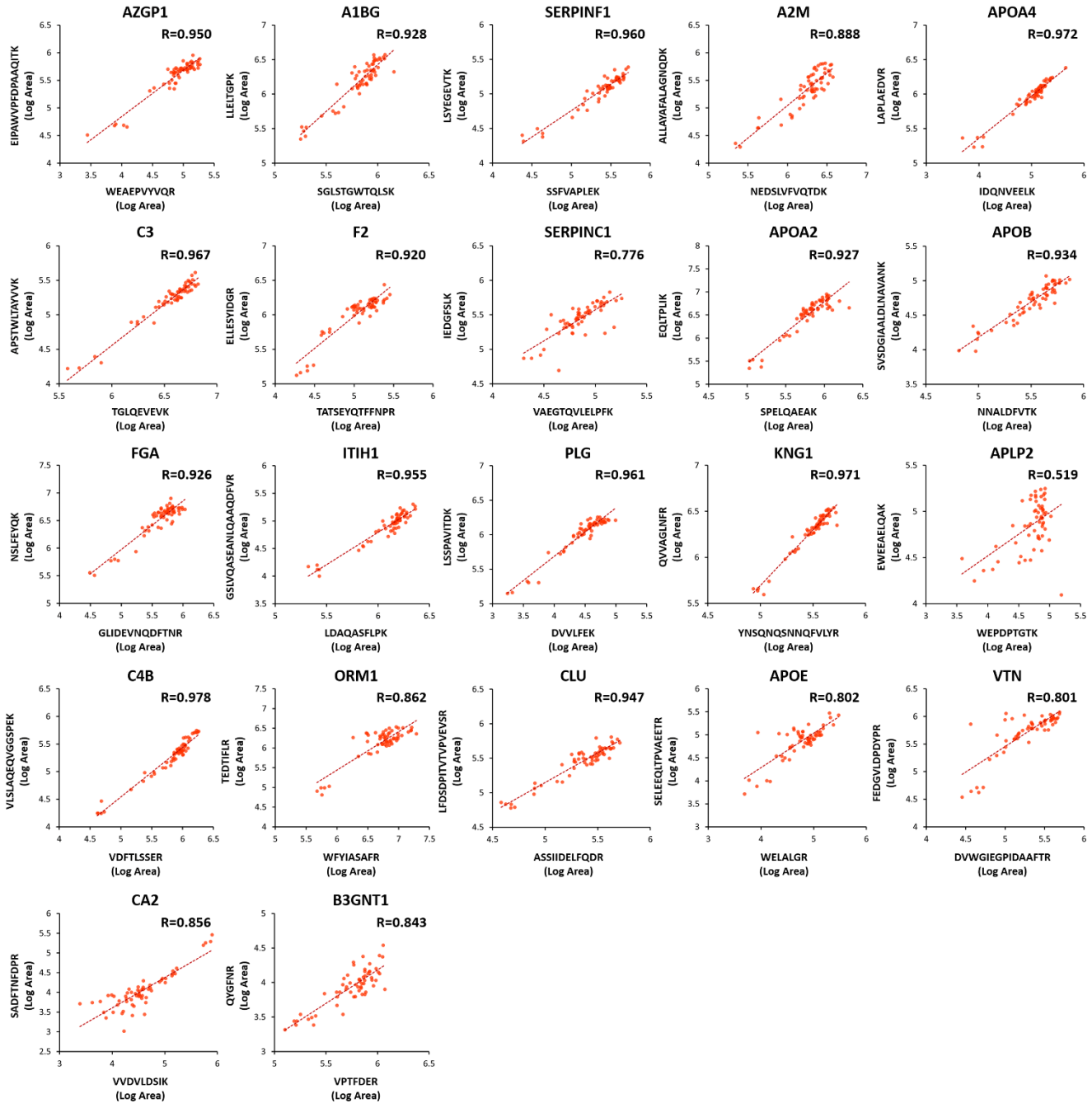
33.3%
Developmental processes
Immunity and defense
Lipid, fatty acid and steroid metabolism
Protein metabolism
Signal transduction

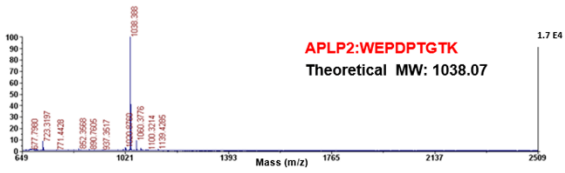

B
REGULATION OF ACTIN CYTOSKELETON
Mi NPDR/No DR
Mo NPDR/No DR
LPS
Chemotactic factor
Bradykinin
Acetylcholine
F2
INS
GF
FN1
PRCD14
RTK
ITG
GPCR
Gαq/11
Sos
FAK
Csr
CrkII
Gβγ
PGD1/3
JNK, p38
MAPK signaling pathway
Gene expression
Raf
MEK
ERK
Ras
c-Src
PI3K
Rac1GEF
Dock180
PIP3
Vav/Tiam1
Adherens junction
IQGAP
Cdc42
Drf3
IRSp53
Mena
Actin polymerization
Arp2/3
F-Actin
Filopodia
NWASP
RhoGEF
GRLF1
Asef
APC
Rho
Rac
PIR121
Nap125
Abi2
HSPC300
WAVE1
TMSB4
PXN
GIT1
PIX
FAK
PFN
Actin polymerization
Focal adhesion assembly
ERM
NHE1
VCL
ACTN
PIP2
PI4P5K
ROCK
IRSp53
WAVE2
Focal adhesion
mDia
MLCP
MLCK
PI4P5K
LIMK
F-Actin
Arp2/3
Lamellipodia
PFN
MLC
CFN
Stabilization of actin
SSH
PIP2
Actin polymerization stress fiber
Actomyosin assembly contraction
F-Actin
MyosinII
Stress fibers
VCL
GSN
Focal complex assembly
Focal adhesion
04810 3/6/13
(c) Kanehisa Laboratories


C
COMPLEMENT AND COAGULATION CASCADES
Mi NPDR/No DR
Mo NPDR/No DR
Coagulation cascade
Extrinsic pathway
Vascular injury
F3
F7
TFPI
F10
F5
F2
Thrombin
PG
Fibrin monomer
Cross-linked fibrin polymer
Clot
Fibrin degradation products
F2R
Platelet, monocyte, lymphocyte, endothelial cells, smooth muscle cells activation, etc.
SERPIND1
CPB2
F11
F9
F8
VWF
Intrinsic pathway
F13
THBD
Degradation
PROC
PROCR
SERPINC1
SERPINA5
A2M
SERPINA1
SERPINB2
Kallikrein-kinin system
PLAT
PLAU
PLAUR
Cell adhesion, migration, proliferation, etc.
Collagen, basement membrane, activated platelets, etc.
F12
PLG
KLKB1
KNG
SERPINF2
Fibrinolytic system
Bradykinin
BDKR
Inflammation, prostaglandin biosynthesis, nitric oxide biosynthesis, etc.
Complement cascade
HF1
Alternative pathway
Zymosan, inulin, etc.
BF
C3
DF
IF
CFHR
DAF
MCP
CD59
C6
C7
C8
C9
C3b/Bb
Lectin pathway
MBL
MASP1/2
C4b/2a
C3
Convertase
C3b
C5
C5b
Membrane attack complex
Cell lysis
Classical pathway
Antibody/ antigen complex
C1Q
C1R
C1S
C2
C4
C4a
C3a
C5a
Mediators
C3AR1
C5R1
Muscle contraction, chemotaxis, phagocyte recruitment, inflammation, etc.
CR2
CR1
B cell receptor signaling pathway
C4BP
C3b/4b, C3b, C3d
SERPING1
04610 4/3/09
(c) Kanehisa Laboratories

Supplementary Fig. 2



Supplementary Fig. 3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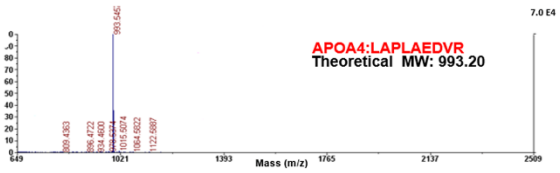
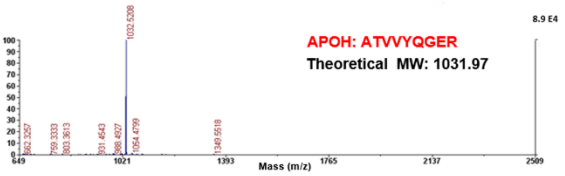
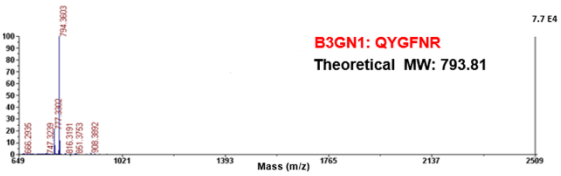
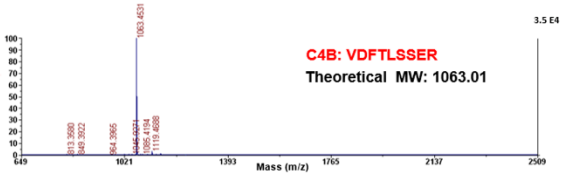
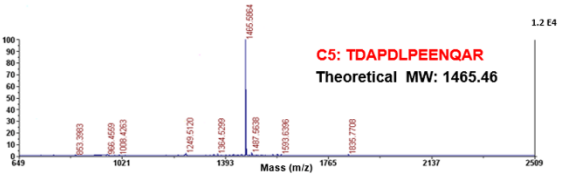
1.7 E4
Mass (m/z)

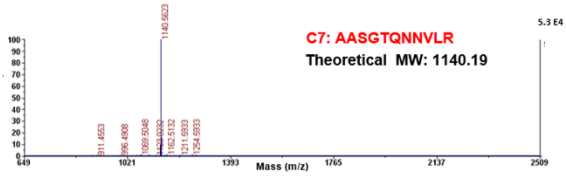

APOA4:LAPLAEDVR
Theoretical MW: 993.20
7.0 E4
Mass (m/z)

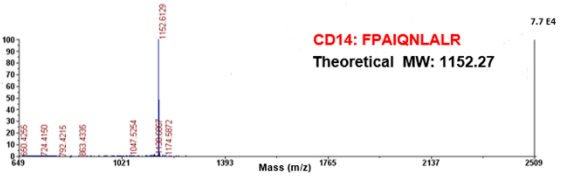

APOH: ATVVYQGER
Theoretical MW: 1031.97
8.9 E4
Mass (m/z)

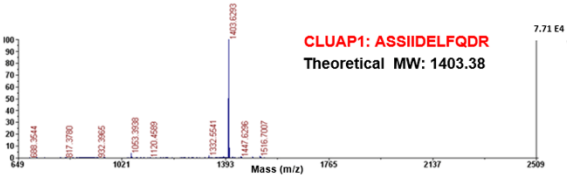

B3GN1: QYGFNR
Theoretical MW: 793.81
7.7 E4
Mass (m/z)

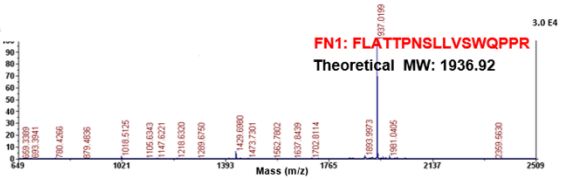

C4B: VDFTLSSER
Theoretical MW: 1063.01
3.5 E4
Mass (m/z)

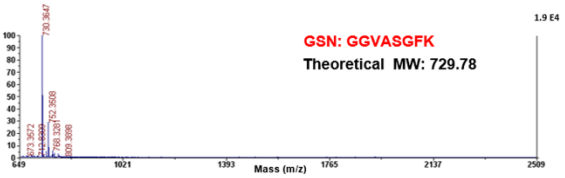

C5: TDAPDLPEENQAR
Theoretical MW: 1465.46
1.2 E4
Mass (m/z)

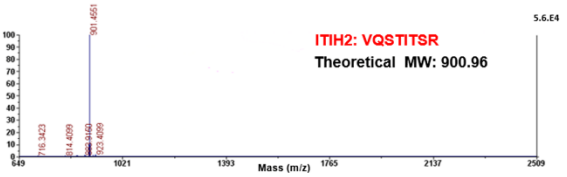

C7: AASGTQNNVLR
Theoretical MW: 1140.19
5.3 E4
Mass (m/z)

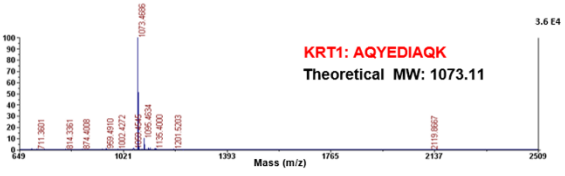

CD14: FPAIQNLALR
Theoretical MW: 1152.27
7.7 E4
Mass (m/z)


CLUAP1: ASSIIDELFQDR
Theoretical MW: 1403.38
7.71 E4
Mass (m/z)


FN1: FLATTPNSLLVSWQPPR
Theoretical MW: 1936.92
3.0 E4
Mass (m/z)


GSN: GGVASGFK
Theoretical MW: 729.78
1.9 E4
Mass (m/z)


ITIH2: VQSTITSR
Theoretical MW: 900.96
5.6.E4
Mass (m/z)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Theoretical MW: 1073.11
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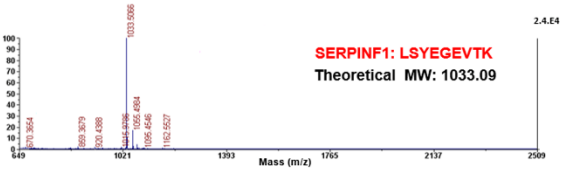
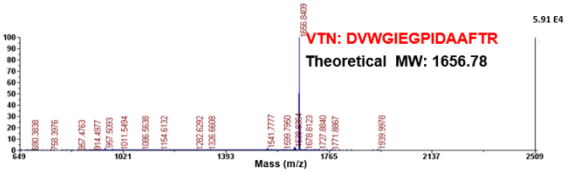
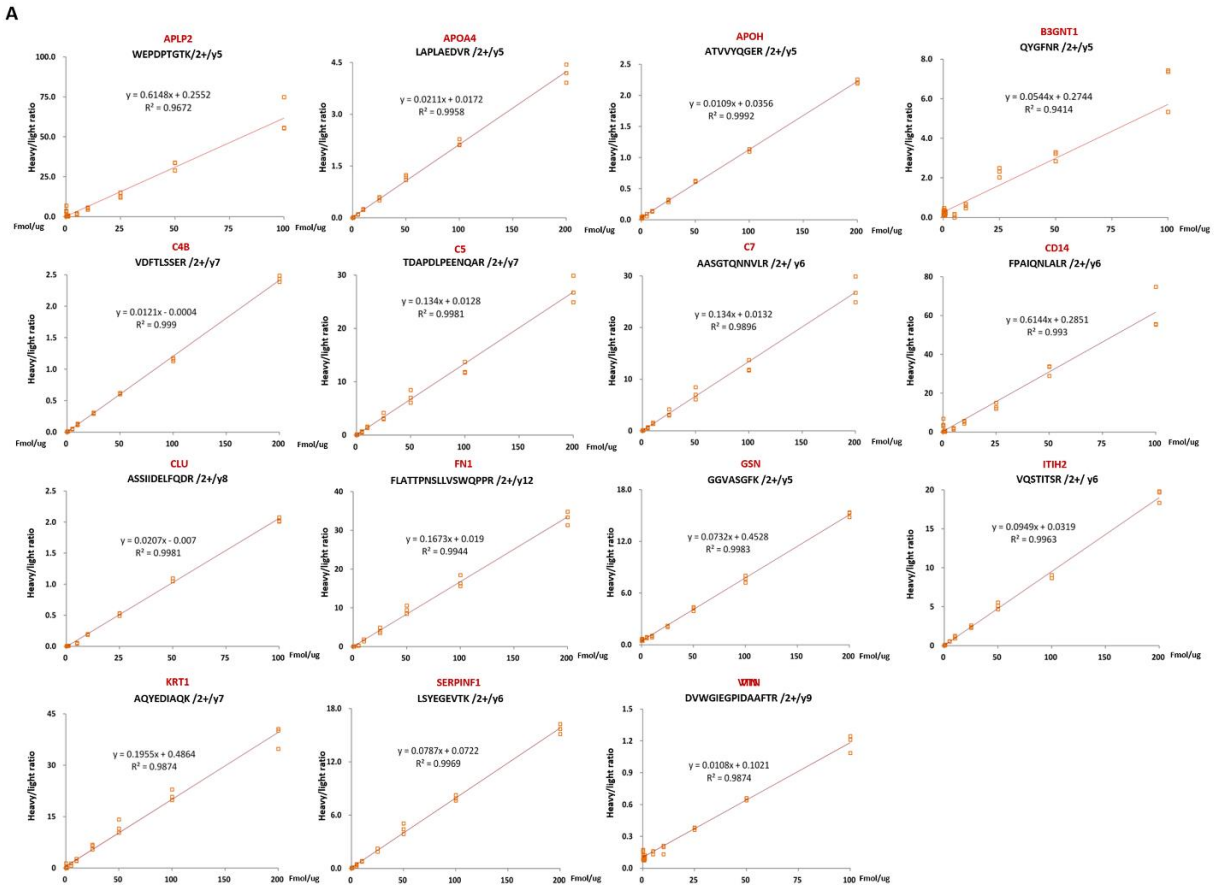
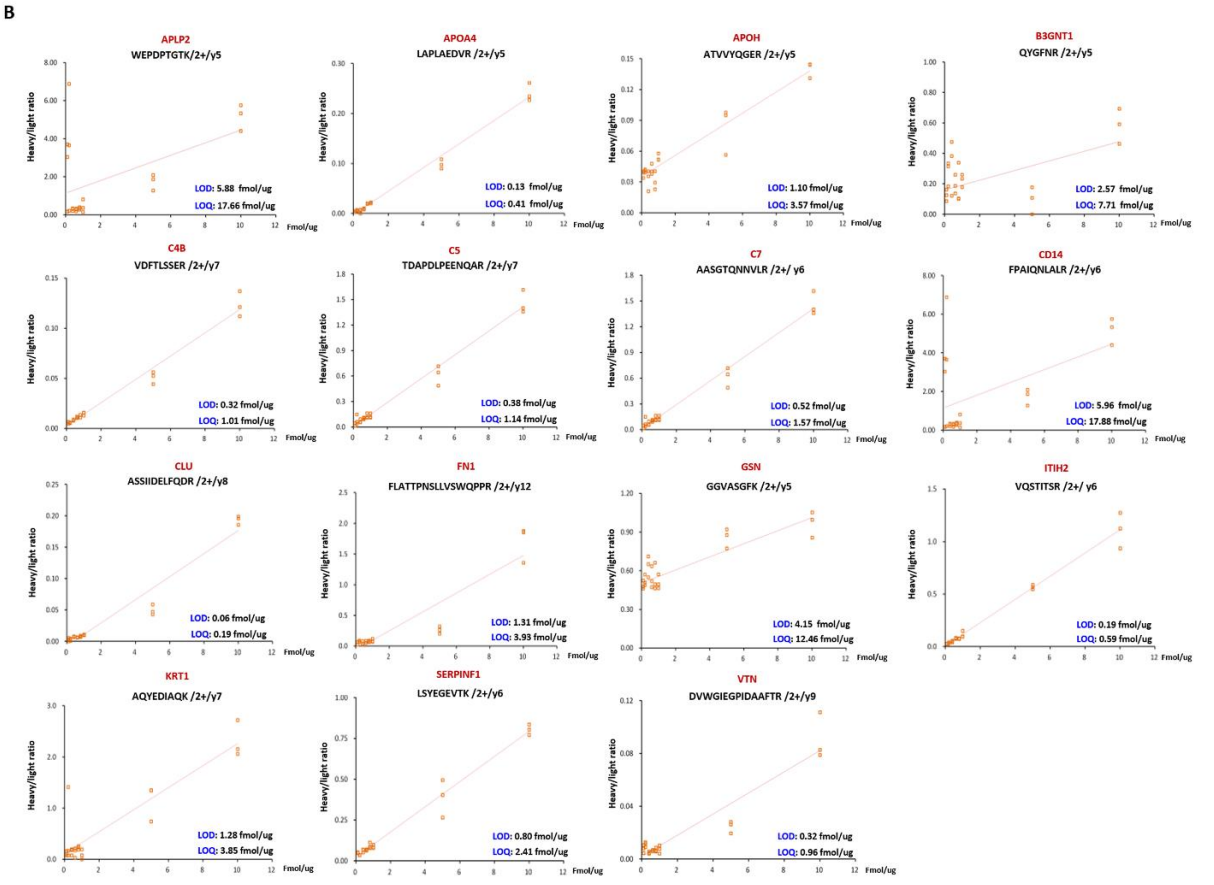
Mass (m/z)


SERPINF1: LSYEGEVTK
Theoretical MW: 1033.09
2.4.E4
Mass (m/z)

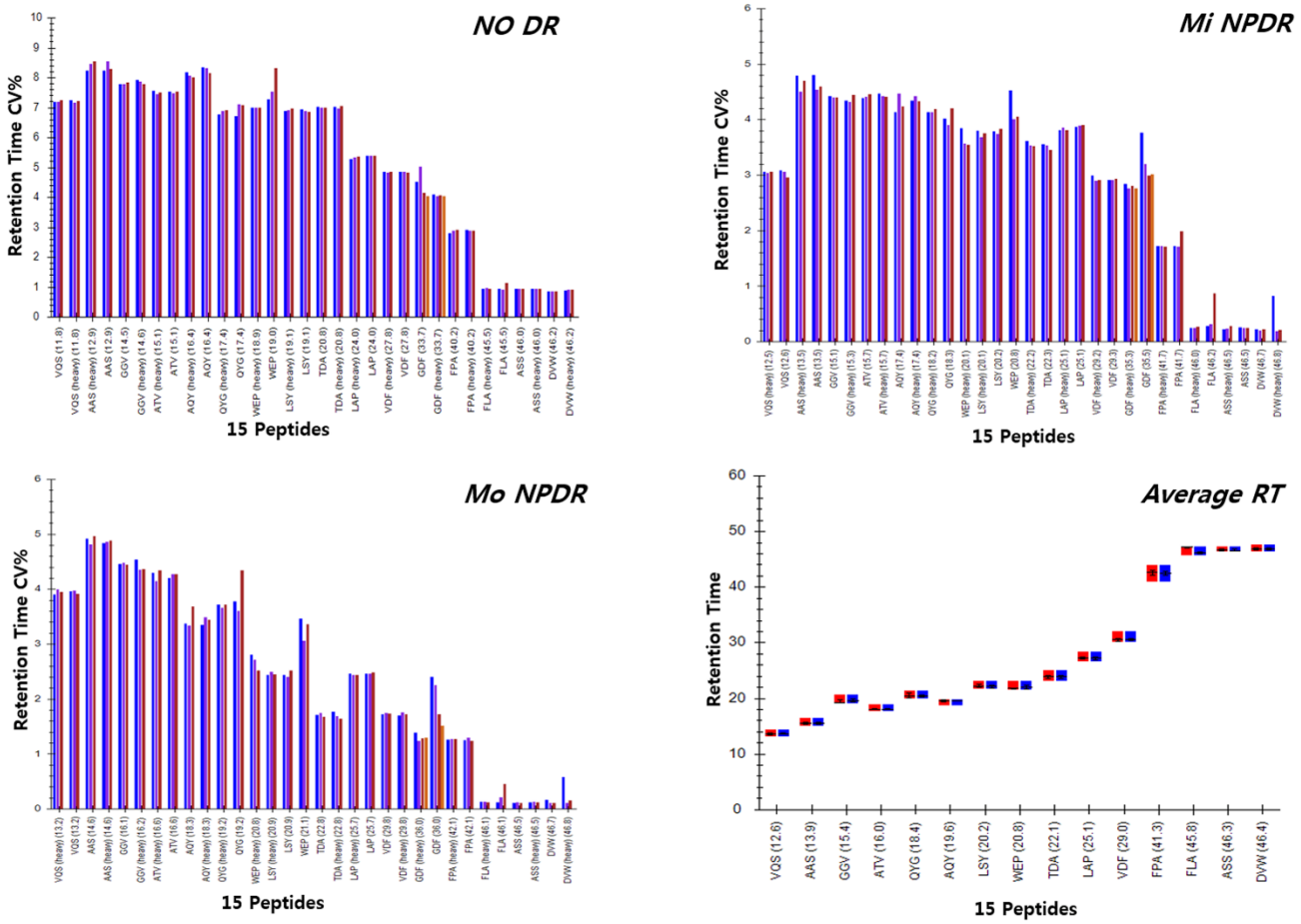

VTN: DVWGIEGPIDAAFTR
Theoretical MW: 1656.78
5.91 E4
Mass (m/z)

Supplementary Fig. 4


A
APLP2
WEPDPTGTK/2+/y5
y = 0.6148x + 0.2552
R² = 0.9672
APOA4
LAPLAEDVR /2+/y5
y = 0.0211x + 0.0172
R² = 0.9958
APOH
ATVVYQGER /2+/y5
y = 0.0109x + 0.0356
R² = 0.9992
B3GNT1
QYGFNR /2+/y5
y = 0.0544x + 0.2744
R² = 0.9414
C4B
VDFTLSSER /2+/y7
y = 0.0121x - 0.0004
R² = 0.999
C5
TDAPDLPEENQAR /2+/y7
y = 0.134x + 0.0128
R² = 0.9981
C7
AASGTQNNVLR /2+/ y6
y = 0.134x + 0.0132
R² = 0.9896
CD14
FPAIQNLALR /2+/y6
y = 0.6144x + 0.2851
R² = 0.993
CLU
ASSIIDELFQDR /2+/y8
y = 0.0207x - 0.007
R² = 0.9981
FN1
FLATTPNSLLVSWQPPR /2+/y12
y = 0.1673x + 0.019
R² = 0.9944
GSN
GGVASGFK /2+/y5
y = 0.0732x + 0.4528
R² = 0.9983
ITIH2
VQSTITSR /2+/ y6
y = 0.0949x + 0.0319
R² = 0.9963
KRT1
AQYEDIAQK /2+/y7
y = 0.1955x + 0.4864
R² = 0.9874
SERPINF1
LSYEGEVTK /2+/y6
y = 0.0787x + 0.0722
R² = 0.9969
VTN
DVWGIEGPIDAAFTR /2+/y9
y = 0.0108x + 0.1021
R² = 0.9874
Heavy/light ratio
Fmol/ug


B
APLP2
WEPDPTGTK/2+/y5
LOD: 5.88 fmol/ug
LOQ: 17.66 fmol/ug
APOA4
LAPLAEDVR /2+/y5
LOD: 0.13 fmol/ug
LOQ: 0.41 fmol/ug
APOH
ATVVYQGER /2+/y5
LOD: 1.10 fmol/ug
LOQ: 3.57 fmol/ug
B3GNT1
QYGFNR /2+/y5
LOD: 2.57 fmol/ug
LOQ: 7.71 fmol/ug
C4B
VDFTLSSER /2+/y7
LOD: 0.32 fmol/ug
LOQ: 1.01 fmol/ug
C5
TDAPDLPEENQAR /2+/y7
LOD: 0.38 fmol/ug
LOQ: 1.14 fmol/ug
C7
AASGTQNNVLR /2+/ y6
LOD: 0.52 fmol/ug
LOQ: 1.57 fmol/ug
CD14
FPAIQNLALR /2+/y6
LOD: 5.96 fmol/ug
LOQ: 17.88 fmol/ug
CLU
ASSIIDELFQDR /2+/y8
LOD: 0.06 fmol/ug
LOQ: 0.19 fmol/ug
FN1
FLATTPNSLLVSWQPPR /2+/y12
LOD: 1.31 fmol/ug
LOQ: 3.93 fmol/ug
GSN
GGVASGFK /2+/y5
LOD: 4.15 fmol/ug
LOQ: 12.46 fmol/ug
ITIH2
VQSTITSR /2+/ y6
LOD: 0.19 fmol/ug
LOQ: 0.59 fmol/ug
KRT1
AQYEDIAQK /2+/y7
LOD: 1.28 fmol/ug
LOQ: 3.85 fmol/ug
SERPINF1
LSYEGEVTK /2+/y6
LOD: 0.80 fmol/ug
LOQ: 2.41 fmol/ug
VTN
DVWGIEGPIDAAFTR /2+/y9
LOD: 0.32 fmol/ug
LOQ: 0.96 fmol/ug
Heavy/light ratio
Fmol/ug

Supplementary Fig. 5



Supplementary Fig. 6

A

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	.449	.212		2.121	.043		
	APOA4	.003	.001	.251	1.946	.062	.307	3.261
	APOH	.002	.001	.313	1.626	.115	.138	7.270
	B3GNT1	-.002	.005	-.073	-.482	.634	.224	4.466
	C4B	.000	.001	.040	.321	.751	.324	3.089
	C5	-.001	.001	-.089	-.723	.475	.338	2.962
	C7	-.031	.012	-.482	-2.666	.013	.156	6.394
	CD14	-.048	.038	-.146	-1.272	.214	.387	2.586
	CLU	.006	.002	.330	2.841	.008	.379	2.642
	GSN	-.012	.007	-.236	-1.622	.116	.242	4.134
	ITIH2	-.007	.005	-.219	-1.475	.151	.233	4.300
	KRT1	-.010	.009	-.103	-1.159	.256	.651	1.537

a. Dependent Variable: Group

B

Variables in the Analysis

Step		Tolerance	F to Remove	Wilks' Lambda
1	ITIH2	1.000	40.152	
2	ITIH2	.857	51.548	.828
	APOA4	.857	15.026	.486
3	ITIH2	.727	9.275	.347
	APOA4	.676	24.066	.460
	C7	.583	9.130	.346
4	ITIH2	.727	6.326	.233
	APOA4	.626	5.597	.229
	C7	.406	20.886	.315
	CLU	.452	13.928	.276

Variables Not in the Analysis

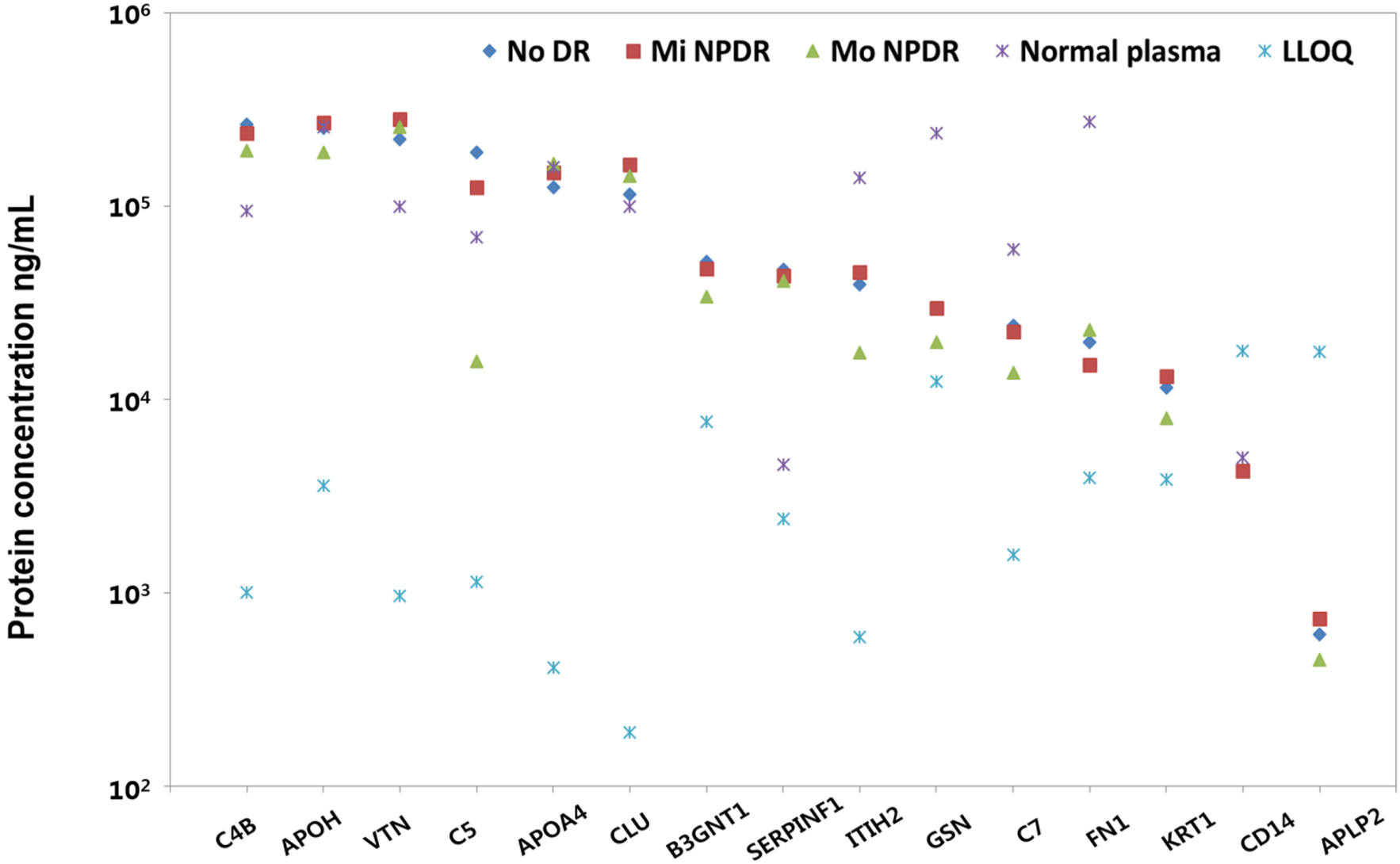
Step		Tolerance	Min. Tolerance	F to Enter	Wilks' Lambda
0	APOA4	1.000	1.000	7.918	.828
	APOH	1.000	1.000	6.952	.845
	B3GNT1	1.000	1.000	16.692	.695
	C4B	1.000	1.000	8.642	.815
	C5	1.000	1.000	16.639	.695
	C7	1.000	1.000	25.408	.599
	CD14	1.000	1.000	7.586	.834
	CLU	1.000	1.000	6.846	.847
	GSN	1.000	1.000	12.836	.748
	ITIH2	1.000	1.000	40.152	.486
	KRT1	1.000	1.000	5.106	.882
1	APOA4	.857	.857	15.026	.346
	APOH	.537	.537	2.470	.456
	B3GNT1	.755	.755	.563	.479
	C4B	.927	.927	.769	.476
	C5	.853	.853	1.504	.467
	C7	.739	.739	2.089	.460
	CD14	.942	.942	.759	.476
	CLU	.843	.843	14.740	.348
	GSN	.716	.716	.028	.486
	KRT1	.967	.967	.599	.478
2	APOH	.357	.357	.477	.341
	B3GNT1	.639	.639	4.199	.310
	C4B	.890	.822	1.964	.328
	C5	.740	.740	5.547	.300
	C7	.583	.583	9.130	.276
	CD14	.785	.714	4.988	.304
	CLU	.648	.648	3.511	.315
	GSN	.592	.592	2.654	.322
	KRT1	.889	.788	2.637	.322
3	APOH	.220	.220	1.735	.263
	B3GNT1	.428	.391	.114	.275
	C4B	.591	.388	.142	.275
	C5	.687	.542	1.997	.261
	CD14	.751	.539	6.682	.232
	CLU	.452	.406	13.928	.197
	GSN	.507	.499	.209	.274
	KRT1	.851	.559	.812	.270
4	APOH	.220	.220	1.670	.188
	B3GNT1	.396	.338	1.408	.189
	C4B	.549	.335	.285	.196
	C5	.670	.398	2.845	.182
	CD14	.739	.402	3.159	.181
	GSN	.504	.373	.386	.195
	KRT1	.829	.403	1.612	.188

IBM SPSS Statistics (IBM Inc., USA, version 20)

- We have performed stepwise MANOVA to determine more reliable candidates for constructing a multi-marker panel.

- **ITIH2**, **APOA4**, **C7**, and **CLU** are finally selected in analysis of stepwise MANOVA

Supplementary Fig. 7


Protein concentration ng/mL
10⁶
10⁵
10⁴
10³
10²
No DR
Mi NPDR
Mo NPDR
Normal plasma
LLOQ
C4B
APOH
VTN
C5
APOA4
CLU
B3GNT1
SERPINF1
ITIH2
GSN
C7
FN1
KRT1
CD14
APLP2