Proteome Dynamics Reveals Pro-inflammatory Remodeling of Plasma Proteome in a Mouse Model of NAFLD.

Authors:

Ling Li, Gurkan Bebek, Stephen F. Previs, Jonathan D. Smith, Rovshan G. Sadygov, Arthur J. McCullough, Belinda Willard, and Takhar Kasumov*

*corresponding author

Supplementary Information

Table of contents

Supplementary Tables (available in separate SI 2-supplementary tables.xlsx)

Supplementary table S1a. Spectral count result of all identified proteins in apoB-depleted plasma in $LDLR^{-/-}$ mice.

Supplementary table S1b.Relative expression of all 175 identified proteins in apoB-depleted plasma in $LDLR^{-/-}$ mice.

Supplementary table S2: The list of proteins only identified in Western diet (WD) or standard diet (SD) group.

Supplementary table S3a. Spectral counts of commonly identified proteins. The proteins were categorized into 4 groups based on the average spectral of proteins from counts in SD group.

S**upplementary table S3b**: The list of 89 proteins presented in all samples from mice on standard (SD, n=6) and Western diet (WD, n=6).

Supplementary table S4. Steady-state metabolic condition during 2H2O-metabolic labeling experiment.

Methods

Label-free quantification.

Since the error observed in the label-free quantification with SC is larger for low-abundant proteins compared to high-abundant proteins, different filtering criteria were used to assess the significance (*P* value) of the observed differences. We used the followings protein abundance categorizations and the significance (*P* value) thresholds associated with fold changes in the proteins abundances (Supplementary table S3-a, S3-b):

Proteins with high abundance: spectral counts >80, NSAF WD/SD ratio ≥1.3 is considered upregulated and NSAF WD/SD ratio ≤0.77 is considered downregulated if $P \leq 0.05$.

Proteins with medium abundance: spectral counts range between 20-80, NSAF WD/SD ratio ≥1.6 is considered upregulated and NSAF WD/SD ratio ≤0.63 is considered down regulated if $P \leq 0.05$.

Proteins with low abundance: spectral counts range between 8-19, NSAF WD/SD ratio ≥1.8 is considered upregulated and NSAF WD/SD ratio ≤0.56 is considered down regulated if $P \leq 0.01$.

Proteins with very low abundance: spectral counts range between 1-7, NSAF WD/SD ratio ≥2 is considered upregulated and NSAF WD/SD ratio ≤0.5 is considered down regulated if $P \leq 0.01$.

Shared peptide handling in label-free quantification

The Scaffold algorithm distributes the spectral counts from shared peptides based on the number of counts from unique peptides. For this purpose, shared peptides are apportioned among proteins according to a weighting function that was described in Scaffoldhelp document.

The weights are assigned by using the following formula:

$$W(p, A) = \frac{PE_{excl}(A)}{\sum_{All(B \supseteq p)} PE_{excl}(B)}$$

Where *W(p, A)* is the weight assigned to shared peptide *p* contained in protein *A* and in other proteins. $PE_{excl}(A)$, the exclusive peptide evidence, is defined as the sum of the probabilities of each exclusive unique valid peptide *X* belonging to protein *A*.

$$PE_{excl}(A) = \sum_{X \subset A} P_X$$

This value is then normalized by the sum of the exclusive peptide evidence for each of the proteins that contain peptide *p*.

Validation of label-free quantification.

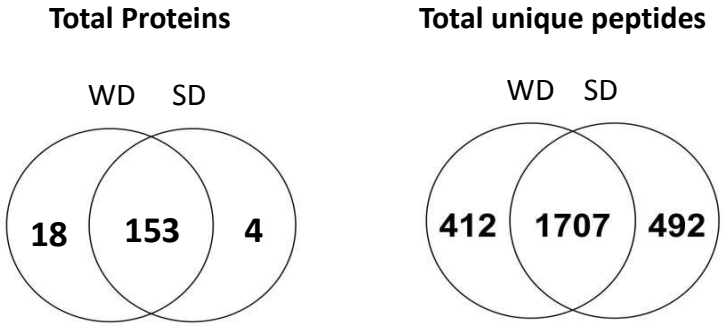
We used 2 different methods to validate spectral-count based quantification. First, we used stable isotope labeled synthetic peptides for absolute quantification of two most abundant HDL proteins, namely apoAI and apoAII. Second, we used the area under the curve of the full scan spectra of selected unique peptides normalized by the chromatographic peak area of a trypsin autolysis peptide, i.e. VATVSLPR, to assess the relative changes in selected protein abundances.

Absolute quantification of apoAI and apoII: The plasma levels of mouse apoAI and apoII were quantified using the ratios of the integrated peak areas of the endogenous mouse apoAI and apoAII peptides VAPLGAELQESAR and THEQLTPLVR to the heavy labeled synthetic

peptides VAPL($^{13}C_6$)GAEL($^{13}C_6$)QESAR ($670.86^{2+}/676.88^{2+}$) and THEQL($^{2}H_{10}$)TPLVR ($597.34^{2+}/602.34^{2+}$), respectively from the full scan spectra.

Chromatographic area quantification: To validate semi-quantitative label-free quantification method, we also used extracted ion chromatograms to quantify the relative abundance peptides unique to the selected proteins. For this purpose, the total ion current for a given peptide was extracted from the full scan MS spectra to construct the ion chromatogram and extracted ion chromatograms (XICs) were aligned by the retention times. Area under the curve under each individually aligned peak was measured, normalized, and compared for relative abundance. This approach was used to confirm spectral counting results for selected proteins that abundances were altered due to a diet-induced NAFLD. We applied the area of reconstructed ion chromatograms method to quantify key proteins of complement pathway. For instance, based on the reconstructed spectral area under the full scan spectra of IILQGSPVVQMAEDAVDGER, a peptide unique to complement C3, we confirmed that complement C3 was suppressed in the WD group. Furthermore, consistent with the label-free quantification results (**Supplementary Figure S4G, S4H)**, complement C9 was more abundant in mice on the WD compared to control animals, as illustrated by the spectral area quantification of the TFDKTDFANWASSLANAPALISQR peptide (**Supplementary Figure S4I, S4J)**, Thus, both isotope dilution and spectral area methods validated our label-free quantification results.

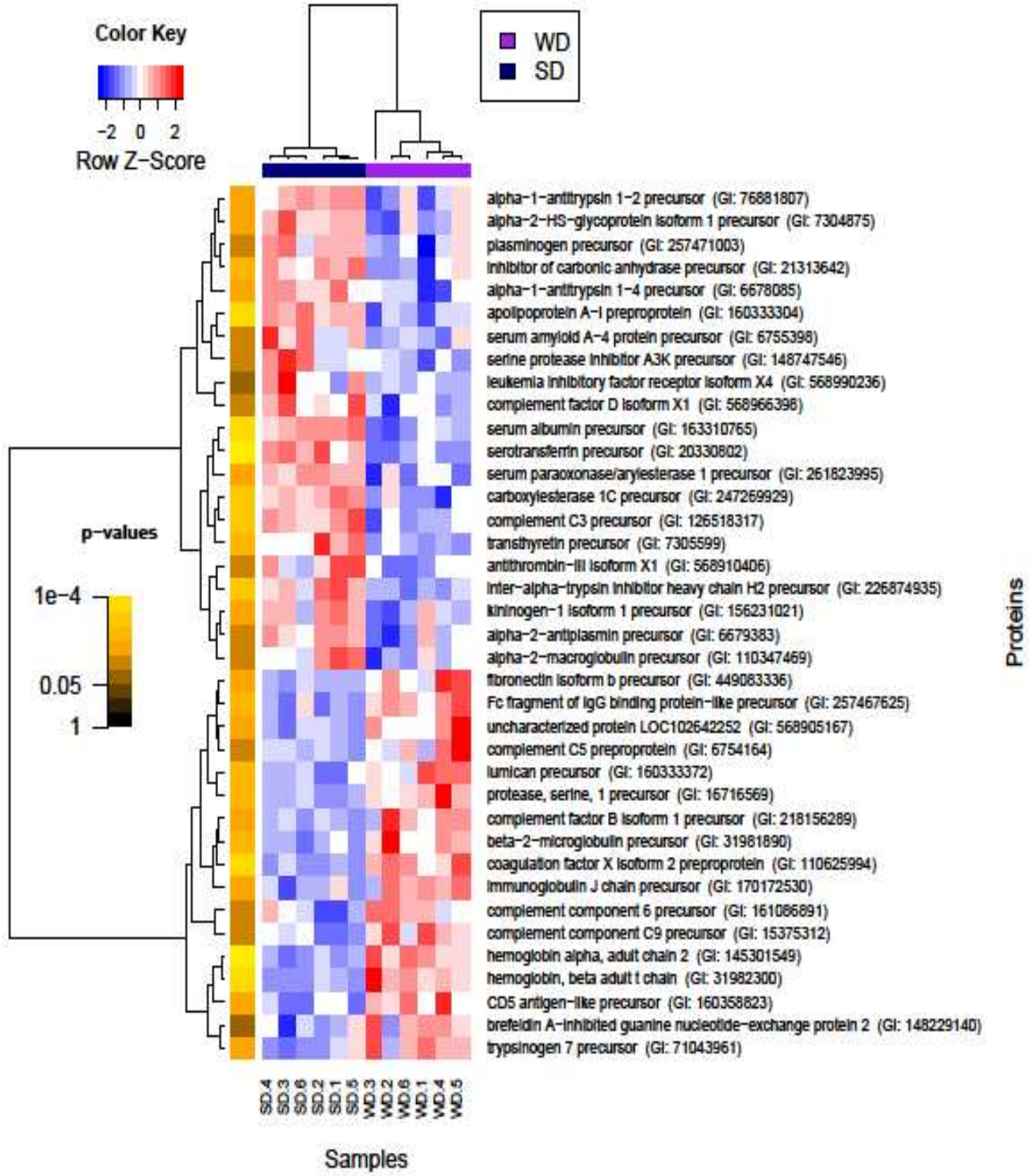
Supplementary figures.



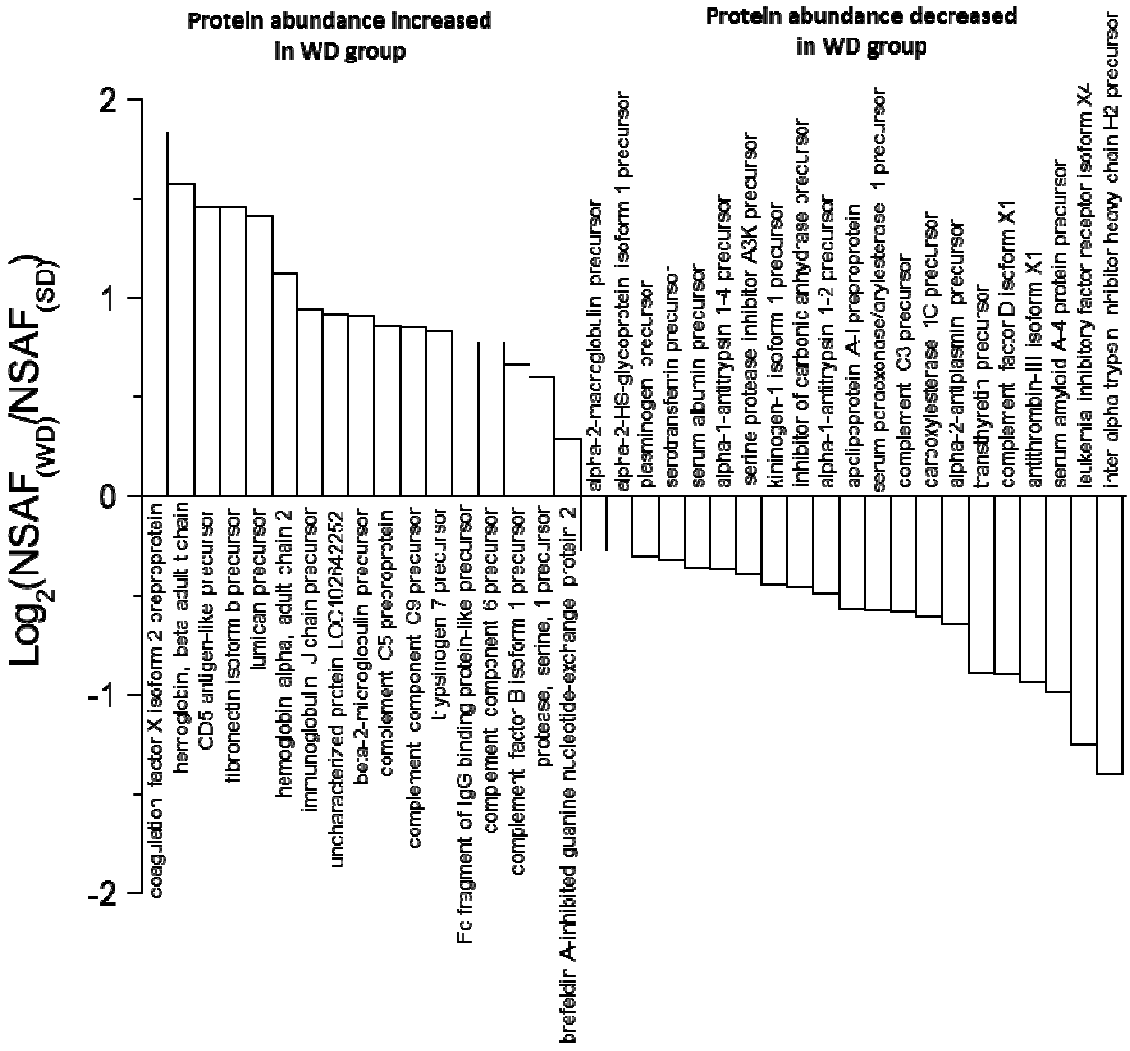
Supplementary Figure S1. Proteomics results from apoB-depleted plasma in LDLR$^{-/-}$ mice (n=6/group).

A: Venn diagram comparing the number of identified proteins identified in Western diet (WD) and standard diet (SD) groups and their relationship; **B:** Venn diagram comparing the number common and distinct of unique peptides identified in WD and SD groups.

A


Color Key
−2 0 2
Row Z−Score
WD
SD
p−values
1e−4
0.05
1
alpha−1−antitrypsin 1−2 precursor (GI: 76881807)
alpha−2−HS−glycoprotein isoform 1 precursor (GI: 7304875)
plasminogen precursor (GI: 257471003)
inhibitor of carbonic anhydrase precursor (GI: 21313642)
alpha−1−antitrypsin 1−4 precursor (GI: 6678085)
apolipoprotein A−I preproprotein (GI: 160333304)
serum amyloid A−4 protein precursor (GI: 6755398)
serine protease inhibitor A3K precursor (GI: 148747546)
leukemia inhibitory factor receptor isoform X4 (GI: 568990236)
complement factor D isoform X1 (GI: 568966398)
serum albumin precursor (GI: 163310765)
serotransferrin precursor (GI: 20330802)
serum paraoxonase/arylesterase 1 precursor (GI: 261823995)
carboxylesterase 1C precursor (GI: 247269929)
complement C3 precursor (GI: 126518317)
transthyretin precursor (GI: 7305599)
antithrombin−III isoform X1 (GI: 568910406)
inter−alpha−trypsin inhibitor heavy chain H2 precursor (GI: 226874935)
kininogen−1 isoform 1 precursor (GI: 156231021)
alpha−2−antiplasmin precursor (GI: 6679383)
alpha−2−macroglobulin precursor (GI: 110347469)
fibronectin isoform b precursor (GI: 449083336)
Fc fragment of IgG binding protein−like precursor (GI: 257467625)
uncharacterized protein LOC102642252 (GI: 568905167)
complement C5 preproprotein (GI: 6754164)
lumican precursor (GI: 160333372)
protease, serine, 1 precursor (GI: 16716569)
complement factor B isoform 1 precursor (GI: 218156289)
beta−2−microglobulin precursor (GI: 31981890)
coagulation factor X isoform 2 preproprotein (GI: 110625994)
immunoglobulin J chain precursor (GI: 170172530)
complement component 6 precursor (GI: 161086891)
complement component C9 precursor (GI: 15375312)
hemoglobin alpha, adult chain 2 (GI: 145301549)
hemoglobin, beta adult t chain (GI: 31982300)
CD5 antigen−like precursor (GI: 160358823)
brefeldin A−inhibited guanine nucleotide−exchange protein 2 (GI: 148229140)
trypsinogen 7 precursor (GI: 71043961)
Proteins
SD.4
SD.3
SD.6
SD.2
SD.1
SD.5
WD.3
WD.2
WD.6
WD.1
WD.4
WD.5
Samples

B

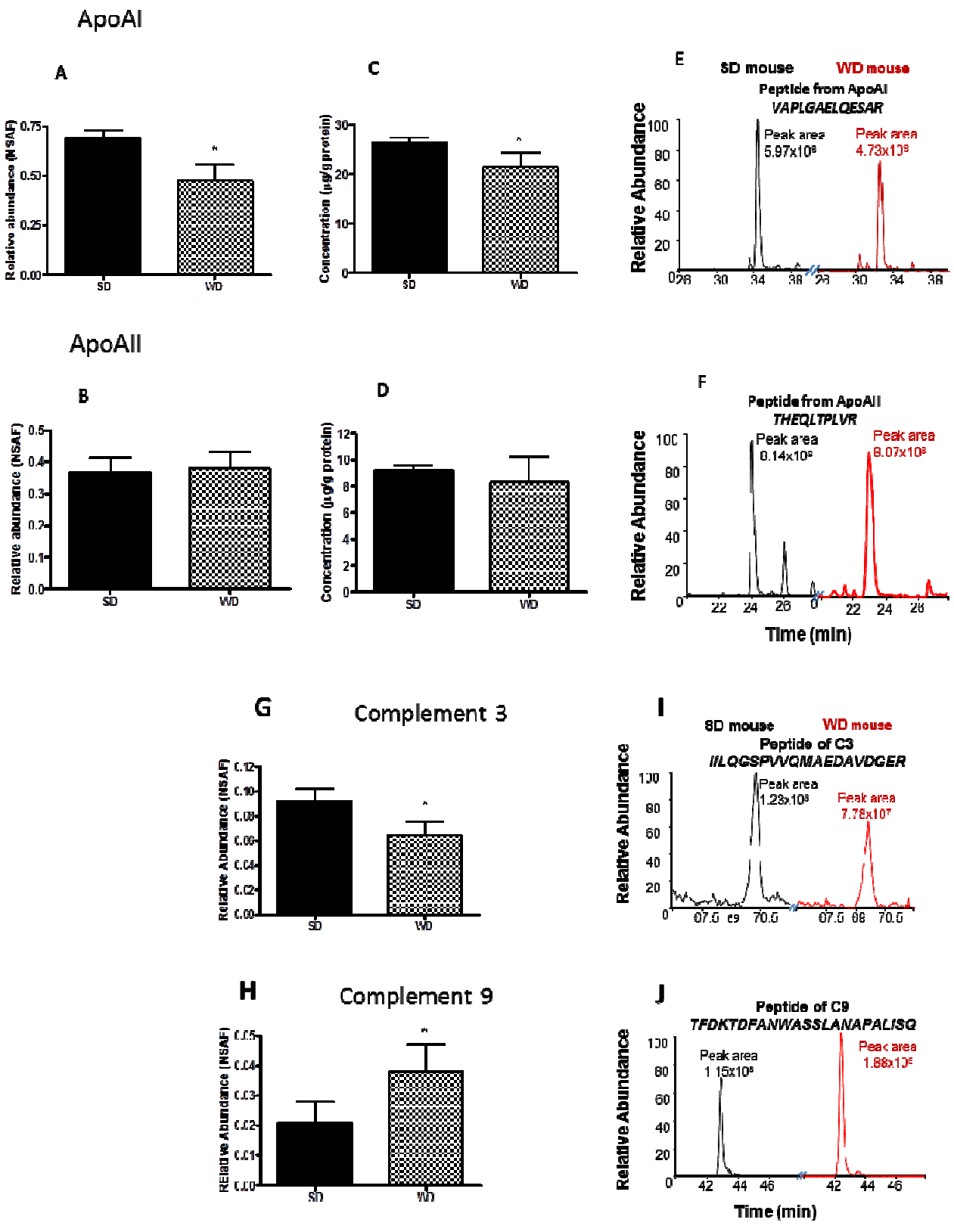


Supplementary Figure S2. Effect of a WD on apoB-depleted plasma proteins' expression. After 12 weeks of a diet experiment, mice were euthanized and plasma proteins were analyzed using label-free protein quantification method (n=6/group).

A: Heatmap of protein abundance differences in each sample. The heatmap is organized based on unsupervised hierarchical clustering. The results are presented row wise (i.e. protein level) z-

scores calculated from NSAF values. Red color indicates increased, and blue color indicates decreased abundances. The top dendogram clusters the two groups of samples (SD vs. WD) and the left dendogram clusters the proteins identified. The black-yellow color bar on the left shows the p-values (t-test) for each protein. The heatmap highlights NSAF changes with diet, and the unsupervised clustering separates the SD and WD samples. The top dendogram splits the proteins into two diet groups. The left dendogram separates the proteins into two major groups where NSAF values are higher in SD compared to WD for the bottom group, and vice versa for the top group. The clustering is generated with R-project (version 3.1.2.) hclust function using Ward-D2 method.

B: Bar figure of 38 significantly changed proteins in label-free quantitation ($P<0.05$).


ApoAI
A
Relative abundance (NSAF)
SD
WD
C
Concentration (µg/g protein)
E
SD mouse
WD mouse
Peptide from ApoAI
VAPLGAELQESAR
Peak area
5.97x10⁸
Peak area
4.73x10⁸
Relative Abundance
ApoAII
B
D
F
Peptide from ApoAII
THEQLTPLVR
Peak area
8.14x10⁸
Peak area
8.07x10⁸
Time (min)
G
Complement 3
I
Peptide of C3
IILQGSPVVQMAEDAVDGER
Peak area
1.23x10⁹
Peak area
7.78x10⁷
H
Complement 9
J
Peptide of C9
TFDKTDFANWASSLANAPALISQ
Peak area
1.15x10⁹
Peak area
1.88x10⁸

Supplementary Figure S3: Validation of label-free quantification of protein abundance.

A&B: Spectral counting based label-free quantitation of apoAI and apoAII expressed as NSAF±SD; label-free quantitation was done using Scaffold software with 1% FDR filter and 95% peptide confidence.

C&D: Absolute quantification of apoAI and apoAII by isotope dilution method expressed as average (μg/g total protein±SD). Protein concentration was quantified by the ratio of endogenous peptide (VAPLGAELQESAR for apoAI and THEQLTPLVR for apoAII) to heavy isotope labeled synthetic peptide internal standards (VAPL(13C6)GAEL(13C6)QESAR for apoAI and THEQL(2H10)TPLVR for apoAII), and the internal standards were quantified using LC-UV based AccQ-Tag method.

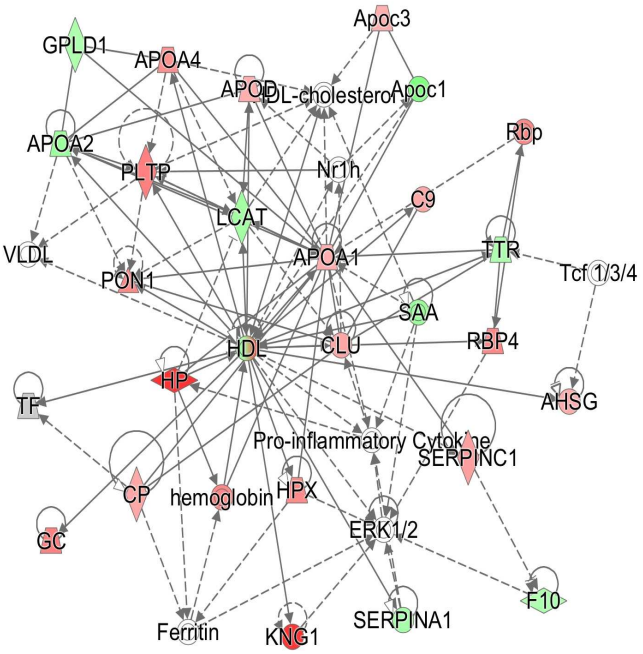
E&F: Chromatographic peak areas of peptide VAPLGAELQESAR from apoAI and peptide THEQLTPLVR from apoAII of a western diet (WD) mouse sample and a standard diet (SD) mouse sample. The peak areas were normalized by the peak area of trypsin autolysis peptide VATVSLPR.

G&H: Spectral counting based label-free quantitation of complement C3 and C9 expressed as NSAF ± SD; label-free quantitation was done using Scaffold software with 1% FDR filter and 95% peptide confidence.

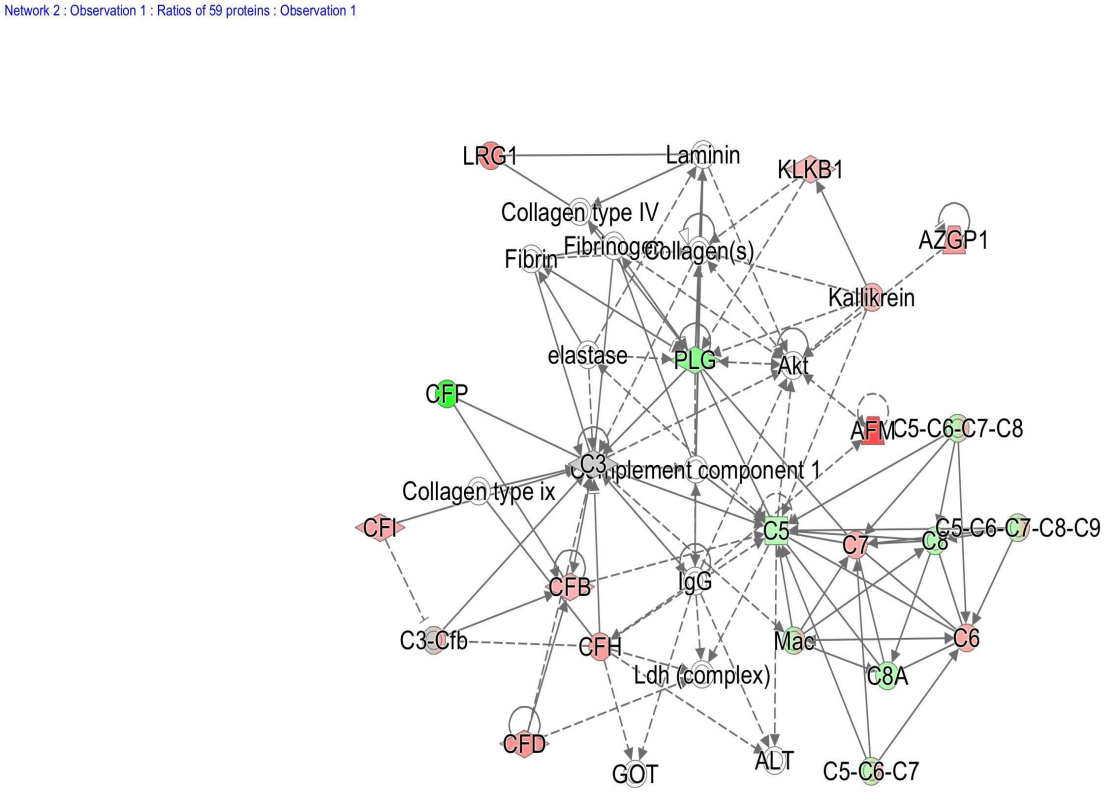
I&J: Representative peak areas under the curve of reconstructed ion chromatograms of peptide IILQGSPVVQMAEDAVDGER from complement C3 and peptide TFDKTDFANWASSLANAPALISQ from complement C9 in plasma from a mouse on WD and a mouse on SD sample. The peak areas were normalized by the peak area of trypsin autolysis peptide VATVSLPR.

A

Network 1 : Observation 1 : Ratios of 59 proteins : Observation 1



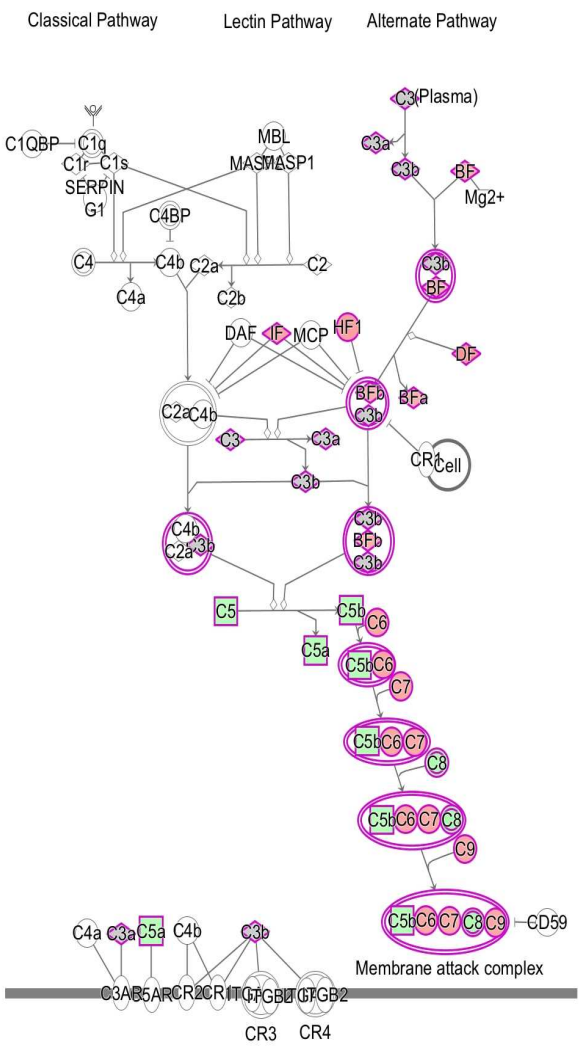
B



Supplementary Figure S4. Ingenuity Pathway Analysis (IPA) of significant expression of HDL network within the dynamic proteome data set. The networks are colored based on WD/SD ratio, where red colors indicate increase in the fractional catabolic rate (FCR) values in WD compared to SD, and green colors represent decreased FCR values in WD compared to SD.

A: HDL proteins involved lipid metabolism is the top ranked network.

B: HDL proteins with immune response function are connected in the second top network.



Supplementary Figure S5. WD activates Complement Pathway. Canonical pathways were scored by Ingenuity Pathway Analysis (IPA), and the Complement System Pathway from this analysis is shown. The networks are colored based on WD/SD ratio, where red colors indicate increase k values in WD compared to SD, and green colors represent decreased k values in WD compared to SD.