



Clusterin Impairs Hepatic Insulin Sensitivity and Adipocyte Clusterin Associates With Cardiometabolic Risk

Diabetes Care 2019;42:466–475 | <https://doi.org/10.2337/dc18-0870>

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OBJECTIVE

Components of the adipose tissue (AT) extracellular matrix (ECM) are recently discovered contributors to obesity-related cardiometabolic disease. We identified increased adipocyte expression of ECM-related clusterin (apolipoprotein J) in obese versus lean women by microarray. Our objective was to determine 1) whether subcutaneous AT adipocyte (SAd) clusterin and serum clusterin are associated with insulin resistance (IR) and known markers of cardiometabolic risk and 2) how clusterin may contribute to increased risk.

RESEARCH DESIGN AND METHODS

We validated increased clusterin expression in adipocytes from a separate group of 18 lean and 54 obese individuals. The relationship of clusterin gene expression and plasma clusterin with IR, cardiovascular biomarkers, and risk of cardiovascular disease (CVD) was then determined. Further investigations in human cultured cells and in aged LDLR^{-/-} mice prone to development of obesity-associated complications were performed.

RESULTS

SAd clusterin correlated with IR, multiple CVD biomarkers, and CVD risk, independent of traditional risk factors. Circulating human clusterin exhibited similar associations. In human adipocytes, palmitate enhanced clusterin secretion, and in human hepatocytes, clusterin attenuated insulin signaling and APOA1 expression and stimulated hepatic gluconeogenesis. LRP2 (megalin), a clusterin receptor, highly expressed in liver, mediated these effects, which were inhibited by LRP2 siRNA. In response to Western diet feeding, an increase in adipocyte clusterin expression was associated with a progressive increase in liver fat, steatohepatitis, and fibrosis in aged LDLR^{-/-} mice.

CONCLUSIONS

Adipocyte-derived clusterin is a novel ECM-related protein linking cardiometabolic disease and obesity through its actions in the liver.

Obesity is associated with a heightened cardiovascular disease (CVD) risk profile that encompasses insulin resistance (IR), type 2 diabetes, hypertension, dyslipidemia, and hepatic steatosis—all components of the well-described metabolic syndrome (1,2). These factors act synergistically to accelerate morbidity and mortality in obesity (3). IR stems from adipocyte lipolysis and lipid deposition into insulin target tissues, as well

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Received 20 April 2018 and accepted 19 December 2018

This article contains Supplementary Data online at <http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc18-0870/-/DC1>.

D.B. and A.B. are co-first authors.

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as a systemic inflammation instigated by adipose tissue (AT) (4). However, identification of specific mediators relating obesity to IR and other CVD risk factors has been unsuccessful.

The adipocyte is increasingly recognized as an important instigator of inflammation in expanding AT (5–7). In obesity, adipocyte enlargement and hypoxia enhance adipocyte production of extracellular matrix (ECM) proteins, leading to the accumulation of collagens and other matrix proteins and, ultimately, fibrosis, which limits AT adaptability (8–11). In addition to AT remodeling, these ECM proteins can enhance diverse functions such as AT inflammation through the influx of immune cells (12,13). We found that expression of clusterin was higher in adipocytes of obese versus lean subjects. Previous studies have suggested that plasma clusterin levels correlate with BMI, inflammation, IR (14), and CVD risk (15–17), and polymorphisms are associated with type 2 diabetes (18). Clusterin is a molecular chaperone that assists with clearance of cellular debris from the ECM via endocytosis and lysosomal degradation (19). Although specific clusterin receptors for many metabolically active tissues, including the liver, have yet to be defined, the most well characterized is LDL receptor–related protein 2 (LRP2). Clusterin binding to LRP2 in the central nervous system affects hypothalamic feeding regulation (20) and leptin signaling (21). Our goal was, therefore, to determine 1) whether adipocytes could secrete clusterin, 2) whether clusterin was associated with insulin action and components of the metabolic syndrome, and, if so, 3) how clusterin impacted insulin target tissues.

RESEARCH DESIGN AND METHODS

Microarray analysis was performed on subcutaneous adipocytes (SAd) obtained from postmenopausal female subjects ($n = 7$ lean and $n = 7$ obese) undergoing elective abdominal surgery. Subjects were not on hormone replacement therapy or medications for hyperlipidemia, hypertension, or diabetes. Demographics and baseline data for group 1 and microarray technology have previously been reported (22). Raw microarray data sets are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database

(accession number GSE44000). Of 428 ECM-related genes in the Gene Set Enrichment Analysis (GSEA) database, genes of interest were identified based on significance, rather than fold change, to reduce bias, since both up- and down-regulated genes could be included in the analyses without independently assigning cutoffs. Also, using P values allowed us to exclude the potential impact of outliers. A PubMed search for the top 48 genes included the gene/protein name and the search terms “insulin resistance” and/or “cardiovascular disease.” The studies were then reviewed individually to assess for published effects on cardiometabolic disease. *CLU* was one of the identified genes, and we performed gene expression, correlation analyses, and blood measurements in an independent group of subjects undergoing elective surgery at The Ohio State University.

Baseline characteristics for this validation group of male and female subjects without diabetes ($n = 18$ lean and $n = 54$ obese) are included in Table 1. Pre-surgery blood samples were obtained prior to induction of anesthesia, after an 8-h overnight fast, and were analyzed for fasting plasma glucose (FPG) using the Glucose Assay Kit and fasting plasma insulin (FPI) (Millipore Sigma, Billerica, MA), triglycerides (TGs), total cholesterol (Tchol), and HDL and LDL cholesterol by ELISA (Millipore Sigma, Billerica, MA). Serum clusterin was measured by ELISA (R&D Systems, Waltham, MA), with a sensitivity of 200 pg/mL, intra-assay coefficient of variation of <10%, and inter-assay coefficient of variation of <12%.

Tissue Procurement, RNA Isolation, and Protein Expression

Subcutaneous AT was biopsied near the umbilicus at either elective bariatric surgery (Roux-en-Y gastric bypass, $n = 30$; sleeve gastrectomy, $n = 24$) in obese subjects or elective surgery (cholecystectomy, $n = 5$; hernia repair, $n = 11$; Heller myotomy, $n = 1$; Nissen fundoplication, $n = 1$) in lean subjects. Patients fasted from midnight prior to surgery; surgeries were performed from 7:00 A.M. to 2:00 P.M. under general anesthesia. Samples were transferred to ice-cold saline and processed within 15 min of tissue excision. SAd and the stromal vascular fraction were isolated as previously described (7), and the adipocytes were leukocyte depleted to achieve <0.1% leukocyte contamination (Supplementary Fig. 1). AT macrophages (ATMs) were then fractionated from the stromal vascular fraction with biotinylated antibodies against CD14 (eBioscience, San Diego, CA) and streptavidin-coupled Dynabeads (Thermo Fisher Scientific, Waltham, MA); there was little contamination of the ATM fraction with adipocytes or T cells (Supplementary Fig. 1). The relative absence of adipocyte precursor cells in the adipocyte fraction was verified by analyzing gene expression of the preadipocyte marker protein delta homolog 1 (preadipocyte factor 1 [*Pref-1*]) (Supplementary Fig. 2).

Quantitative RT-PCR (qRT-PCR) was performed as previously described (6). In brief, RNA was isolated using Direct-zol RNA MiniPrep kit (Zymo Research), reverse transcribed with High-Capacity

Table 1—Characteristics of the study population undergoing qRT-PCR (group 2) analyses

	Validation study population	
	Lean ($N = 18$)	Obese ($N = 54$)
n female/ n male (% female)	5/13 (27)	31/23 (57)
Age (years)	44.9 ± 12.4	44.2 ± 10.4
BMI (kg/m^2)	23.1 ± 1.6	49.7 ± 8.7 ^a
SBP (mmHg)	126.9 ± 19.6	140.2 ± 14.8 ^a
DBP (mmHg)	75.8 ± 10.2	78.6 ± 10.6
Plasma parameters		
Tchol (mg/dL)	182.8 ± 32.6	167.2 ± 31.2
HDL cholesterol (mg/dL)	58.0 ± 14.6	43.4 ± 9.4 ^a
LDL cholesterol (mg/dL)	104.0 ± 34.5	95.3 ± 29.9
TGs (mg/dL)	104.9 ± 89.9	144.7 ± 53.9
FPG (mg/dL)	85.5 ± 13.0	114.2 ± 34.7 ^a
FPI ($\mu\text{IU}/\text{mL}$)	7.3 ± 6.0	22.6 ± 14.1 ^a
HOMA-IR	1.9 ± 1.7	5.7 ± 4.9 ^a

Data are means ± SD unless stated otherwise. ^a $P < 0.05$ in lean vs. obese patients.

cDNA Reverse Transcription kits (Thermo Fisher Scientific), and analyzed by using gene-specific primer/probe sets with values normalized to *PPIA* expression. RNA was analyzed for relative expression of *CLU*. Gene expression values are shown as the fold change, defined by $2^{-\Delta\Delta CT}$ as recommended by the manufacturer and according to standard protocol (6,7,23). Adipocyte protein levels of clusterin were analyzed by Western blotting of visceral adipocytes (VAd) obtained from lean ($n = 8$) and obese ($n = 6$) patients as previously described (5,6).

Calculations

IR was determined by HOMA of IR (HOMA-IR) (24). Ten-year CVD risk was calculated according to the Framingham Risk Score algorithm (25).

Statistical Analyses

Normality was determined by the Kolmogorov-Smirnov criteria. Gene expression and other variables between lean and obese groups were compared by Student *t* test (normally distributed data) or Mann-Whitney *U* test (nonnormally distributed data). Pearson/Spearman correlations were calculated to assess associations. Multivariable analysis with independent variables of age, sex, BMI, smoking status, and individual gene expression values was performed in stepwise fashion with predicted adjusted values calculated by SPSS, version 24.

In Vitro Treatment of Cultured Human Hepatocytes, Skeletal Muscle Cells, and Adipocytes

Human preadipocytes (ZenBio, Research Triangle Park, NC) were differentiated according to the manufacturer's instructions. Secreted clusterin in conditioned medium of human cultured adipocytes was analyzed by ELISA, following treatment with palmitate (200 $\mu\text{mol/L}$), adiponectin (20 $\mu\text{g/mL}$), IFN γ (2 ng/mL), interleukin (IL)-6 (50 ng/mL), IL-33 (50 ng/mL), and TGF β 1 (10 ng/mL).

For examination of clusterin's effects on protein phosphorylation of AKT, human liver HepG2 cells (ATCC, Manassas, VA) and skeletal muscle cells and adipocytes (ZenBio) were cultured according to manufacturers' protocols and pretreated in quiescent medium (0.5% FBS) with recombinant human clusterin protein (concentrations 0.1, 0.5, and 5.0 $\mu\text{g/mL}$) (BioLegend, San Diego, CA)

for 20 h before incubation with human insulin (50 nmol/L) for 30 min. Human HepG2 cells were maintained in Eagle's Minimum Essential Medium (ATCC) (26). Human adipocytes were maintained in adipocyte maintenance medium containing DMEM, HEPES (pH 7.4), FBS, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin, and amphotericin B; stored at -20°C ; and used by the expiration date. Human skeletal muscle myoblasts were maintained in skeletal muscle cell growth medium containing DMEM, FBS, BSA, fetuin, human epidermal growth factor, dexamethasone, human insulin, penicillin, streptomycin, and amphotericin B; stored at -20°C ; and used within the expiration date. Protein extraction and separation, immunoblotting, and visualization were performed as previously described (6). Phosphorylated AKT (S473 and T308) and total AKT antibodies were purchased from Cell Signaling Technology.

qRT-PCR was performed in triplicate on samples from cultured HepG2 cells as previously described (6). RNA was analyzed for relative expression of *APOA1* (apolipoprotein A1), *AGT* (angiotensinogen), *PON1* (paraoxonase-1), key genes regulating gluconeogenesis (*PCK1* and -2 [phosphoenolpyruvate carboxykinase 1 and 2], *GCK* [glucokinase], and *PKLR* [pyruvate kinase isozyme R]), and *SREBP1*. Relative expression of putative clusterin receptors including *LRP2* (megalin), *LRP8* (ApoER2), *TGFBR1* (TbR1), *TGFBR2* (TbR2), and *VLDLR* was determined in cultured HepG2 cells, skeletal muscle cells, and human adipocytes.

For LRP2 knockdown experiments, HepG2 cells were cultured according to manufacturers' protocols and pretreated in quiescent medium (0.5% FBS). After reaching 70–80% confluence, cells were transfected using Lipofectamine RNAiMAX Transfection Reagent (catalogue number 13778030; Invitrogen) with 50 nmol/L LRP2 SMARTpool siRNA (L-012673-00-0005; Dharmacon) or control nontargeting siRNA (D-001810-10-05; Dharmacon) in growth media (Eagle's minimal essential medium, 10% FBS, penicillin, and streptomycin). For phosphorylated AKT experiments, siRNA media was removed after 48 h and replaced with Basal Medium Eagle with 0.5% FBS, penicillin, streptomycin, and clusterin (5 $\mu\text{g/mL}$) and treated for 24 h. Insulin (50 nmol/L) was added for 30 min and

protein lysates were collected for Western blot analysis as described above. For quantitative PCR, after 48 h clusterin was added directly to siRNA media and pretreated for 4 h, and then 50 nmol/L insulin was added and cells were incubated for 20 h. Zymo RNA Lysis Buffer (Zymo Research) was added, cells were collected, and qRT-PCR was carried out as previously described (6).

Investigation of Nonalcoholic Steatohepatitis in Mice

Lean *LDLR*^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME), group housed under a 12-h light/dark cycle, and fed standard chow (8904; Harlan Teklad). The age of all mice at the time of sacrifice was \sim 72 weeks. For induction of weight gain, steatosis, and nonalcoholic steatohepatitis (NASH), middle-aged mice (52 weeks) were switched from chow to a Western diet (WD) (40% kcal fat) (D12079B; Research Diets, New Brunswick, NJ) for 6, 8, 10, and 11 weeks prior to the study end point (27). The control mice were maintained on chow diet throughout the duration of the study. All animal procedures were conducted in specific-pathogen-free facilities at The Ohio State University Wexner Medical Center and approved by The Ohio State University Institutional Animal Care and Use Committee. At time of tissue harvest, the epididymal fat pads and livers were removed. Liver was formalin fixed en bloc. Histological analysis was performed to determine steatosis, lobular inflammation, ballooning degeneration, and fibrosis by an unbiased liver pathologist (M.Y.), and NAFLD Activity Score (NAS) was calculated (28). Adipocytes were harvested and subjected to gene expression analyses (6).

RESULTS

Adipocyte Gene and Protein Expression of Clusterin Is Upregulated in Obesity

Out of the top differential genes on microarray, we identified clusterin as one of the proteins previously reported to influence cardiovascular/metabolic risk. Clusterin overexpression was validated by qRT-PCR in an independent cohort of healthy lean ($n = 18$) and obese ($n = 54$) individuals, with obese subjects displaying many metabolic syndrome components but without diabetes (Table 1). The obese subjects had higher BMI

($P < 0.001$), FPG ($P = 0.037$), FPI ($P = 0.004$), systolic blood pressure (SBP) ($P = 0.022$), Tchol-to-HDL cholesterol ratio ($P = 0.045$), and HOMA-IR ($P = 0.007$) compared with lean participants. There was a trend for higher TGs ($P = 0.122$) in the obese subjects. The obese subjects had lower HDL cholesterol ($P < 0.001$) but no difference in LDL cholesterol ($P = 0.504$) or Tchol ($P = 0.221$). *CLU* gene expression was upregulated in SAd (fold change 2.4 ± 0.2 vs. 1.2 ± 0.2 , $P = 0.004$), but not subcutaneous AT macrophage (SATM), of obese versus lean patients (Fig. 1A). Similar to the observed increase in *CLU* gene expression (Fig. 1A), protein levels of clusterin were substantially higher in SAd of obese versus lean humans ($P = 0.037$) (Fig. 1B).

Adipocyte Gene Expression and Serum Clusterin Are Associated With IR and Impair Hepatic Insulin Sensitivity

To detect whether clusterin impacts metabolism, we analyzed the relationship of adipocyte and ATM clusterin mRNA expression with HOMA-IR. Only SAd *CLU*, but not SATM, was significantly related to HOMA-IR and FPI (Fig. 1E and F), with an increase in adipocyte *CLU* expression in insulin-resistant (HOMA-IR ≥ 3) compared with insulin-sensitive (HOMA-IR < 3) subjects (2.5 ± 0.3 vs. 1.4 ± 0.2 , $P = 0.012$) (Fig. 1D). Multiple regression was performed to predict HOMA-IR based on adipocyte *CLU* expression, age, sex, BMI, and smoking status (F(5, 3.118), $P = 0.014$, $r = 0.654$), with only *CLU* significantly adding to the prediction model ($\beta = 0.492$, $P = 0.009$). When a similar analysis was performed using FPI (F(5, 5.957), $P = 0.015$, $r = 0.652$), only *CLU* was significant ($\beta = 0.460$, $P = 0.013$). In contrast, *CLU* SATM gene expression was not related to IR. We measured circulating clusterin levels, which were significantly higher in obese compared with lean subjects (Fig. 1C), positively related to BMI ($P = 0.045$), and trended to rise in association with HOMA-IR (Fig. 1G), suggesting that serum levels rose in parallel with SAd gene expression. There was no significant difference based on sex in *CLU* adipocyte expression (females 2.0 ± 1.2 vs. males 2.1 ± 1.4 ; $P = 0.911$) or serum clusterin (females $362,622 \pm 94,330$ pg/mL vs. males $326,115 \pm 72,483$ pg/mL; $P = 0.112$) (Supplementary Fig. 3).

Adipocyte Gene Expression of Clusterin and Serum Clusterin Are Independently Associated With Validated Biomarkers of CVD Risk

SAd *CLU* was significantly related to TG (Fig. 1H) and Tchol-to-HDL cholesterol ratio (Fig. 1I) and inversely associated with HDL (Fig. 1J), even after multivariable adjustment for cardiovascular risk factors (age, sex, BMI, smoking status) ($\beta = 0.341$, $P = 0.050$). In addition, SAd *CLU* was significantly related to SBP (Fig. 1N) and diastolic blood pressure (DBP) (Fig. 1O), even after multivariate adjustment ($\beta = 0.349$, $P = 0.039$). SAd *CLU* was also related to 10-year risk of CVD (composite myocardial infarction and stroke) (Fig. 1P) and 10-year risk of cardiovascular-related mortality ($r = 0.435$, $P = 0.013$).

We next determined whether circulating clusterin had a similar relationship with cardiovascular risk. Serum clusterin positively correlated with TGs and Tchol-to-HDL cholesterol ratio (Fig. 1K and L). When we accounted for age, sex, BMI, and smoking status, serum clusterin remained the only significant positive predictor of these risk factors ($\beta = 0.440$, $P = 0.015$, and $\beta = 0.461$, $P = 0.014$, respectively). We also observed a negative association between serum clusterin and HDL cholesterol (Fig. 1M) that exhibited a trend after multivariable adjustment ($\beta = -0.318$, $P = 0.076$). In contrast, serum clusterin concentrations were not associated with cardiovascular risk/mortality, SBP, or DBP.

For understanding of how clusterin may affect cardiovascular risk, HepG2 cells, a human hepatoma cell line, were treated with clusterin. In the presence of insulin, clusterin decreased *APOA1* expression (Fig. 2D), a major component of HDL cholesterol and a biomarker of reduced myocardial infarction risk (29), without altering expression of *AGT*, a regulator of blood pressure, or *PON1*, an antioxidant factor in HDL (data not shown).

Clusterin Secretion Is Increased in Response to Palmitate

To determine whether clusterin is secreted from adipocytes, we treated cultured human adipocytes with factors known to influence IR (30). *CLU* protein levels in the cell lysates were measured by Western blot, and secretion was determined by ELISA of the adipocyte cell media. *CLU* gene expression and protein levels increased with addition

of palmitate (Fig. 2A and B) but not with adiponectin or proinflammatory cytokines (IFN γ , IL-6, IL-33, TGF β 1 [data not shown]). These data indicate that the adipocyte expresses the clusterin gene and protein and that adipocyte clusterin secretion is regulated by palmitate.

Recombinant Clusterin Impairs Hepatic Insulin Sensitivity Through the LRP2 Receptor

To determine whether clusterin inhibits insulin action, we treated cultured HepG2 cells, human adipocytes, and human skeletal muscle cells with insulin with or without recombinant clusterin. In HepG2 cells, clusterin treatment decreased insulin-induced AKT phosphorylation (Fig. 2C) but had no effect on skeletal muscle cells or adipocytes to inhibit insulin action (Supplementary Fig. 4). Clusterin also augmented genes representing enzymatic steps in hepatic gluconeogenesis, including a dose-dependent increase in insulin-suppressed expression of *GCK* (converts glucose to glucose-6-phosphate) and *PKLR* (upregulates gluconeogenesis and reduces glycolysis/glycogen synthesis) (31) (Fig. 2D), further suggesting that clusterin antagonizes insulin action. To identify potential receptor(s) responsible for the effects of clusterin on insulin signaling, we next measured gene expression of known clusterin receptors in HepG2 cells, skeletal muscle cells, and VAd and SAd. There was markedly higher expression of *LRP2* (megalin) in HepG2 cells compared with human skeletal muscle or VAd and SAd (Fig. 2E), which demonstrated higher expression levels of *LRP8*, *TGFBR1* and -2, and *VLDLR*, which are other putative clusterin receptors (Supplementary Fig. 5). To determine whether *LRP2* mediated the effects of clusterin, HepG2 cells were transfected with nontargeted scrambled siRNA (scRNA) or *LRP2*-targeted siRNA. The siRNA decreased *LRP2* by 60% compared with scRNA in the presence of clusterin, which is known to stimulate *LRP2* expression (32) (Fig. 2F). Clusterin inhibited insulin signaling in cells treated with scRNA but not in samples treated with *LRP2* siRNA (Fig. 2G). Similarly, expression of *APOA1*, *GCK*, *PCK1*, and *SREBP1* was altered in the presence of scRNA but not *LRP2* siRNA (Fig. 2H). These data suggest that clusterin binds to *LRP2* to inhibit insulin signaling and alter gene expression in human liver cells.

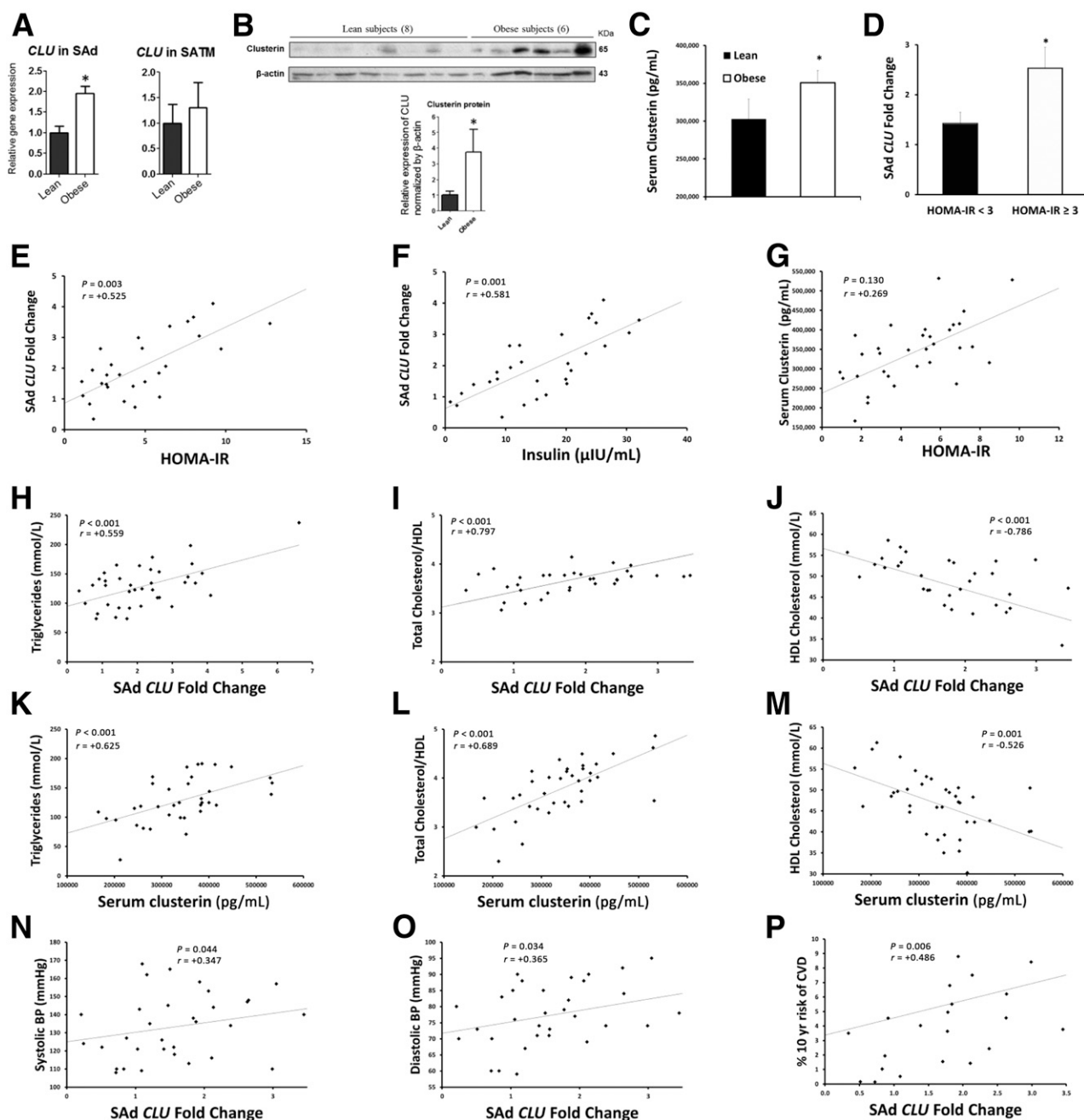


Figure 1—A: SAd and SATM gene expression of *CLU* in lean and obese subjects. B: Clusterin protein expression by Western blotting in human lean and obese subjects. C: Serum clusterin concentrations in lean and obese subjects by Student *t* test. D: SAd fold change in *CLU* in patients with HOMA-IR <3 or ≥ 3 by Student *t* test. * $P < 0.05$. Multiple linear regression analyses between adipocyte gene expression of *CLU* by qRT-PCR vs. adjusted HOMA-IR (E) and fasting insulin (F). G: Multiple linear regression analyses between serum clusterin concentrations (pg/mL) vs. adjusted HOMA-IR. Multiple linear regression analyses between SAd gene expression and serum clusterin by qRT-PCR vs. adjusted TGs (H and K), the ratio of Tchol to HDL cholesterol (I and L), and HDL cholesterol levels (J and M). Covariates in regression model included adipocyte *CLU* expression or serum clusterin and age, sex, BMI, and smoking status. Values on the y-axis are adjusted for dependent variables from the regression model. Patients on HMG-CoA reductase inhibitors (i.e., statins) or other antihyperlipidemic medications were excluded from analyses. Correlation analyses (Spearman) between adipocyte gene expression of *CLU* by qRT-PCR vs. SBP (N), DBP (O), and 10-year risk of cardiovascular event (P). Patients on antihypertensive medications at the time of measurement were excluded from analyses in G and H, and patients with known CVD were excluded from analyses in I. Ten-year risk score calculated according to the Framingham Risk Score algorithm (25). BP, blood pressure; yr, year.

Adipocyte Gene Expression of Clusterin Is Temporally Related to Hepatic Steatosis

In order to determine whether SAd *CLU* is associated with liver fat accumulation and NASH, we examined the time course

of SAd *CLU* expression, percent liver fat, and degree of steatohepatitis in aged (>65 weeks old) LDLR^{-/-} mice fed WD, a model of NASH (33). Body weight (Fig. 3A) and percent body fat (Fig. 3B) increased progressively over time with

WD. Serum clusterin (Fig. 3D) and adipocyte clusterin expression (Fig. 3E) were increased in WD-fed versus chow-fed mice at 6, 8, 10, and 11 weeks of WD, plateauing at 8 weeks, and temporally followed the same pattern as the

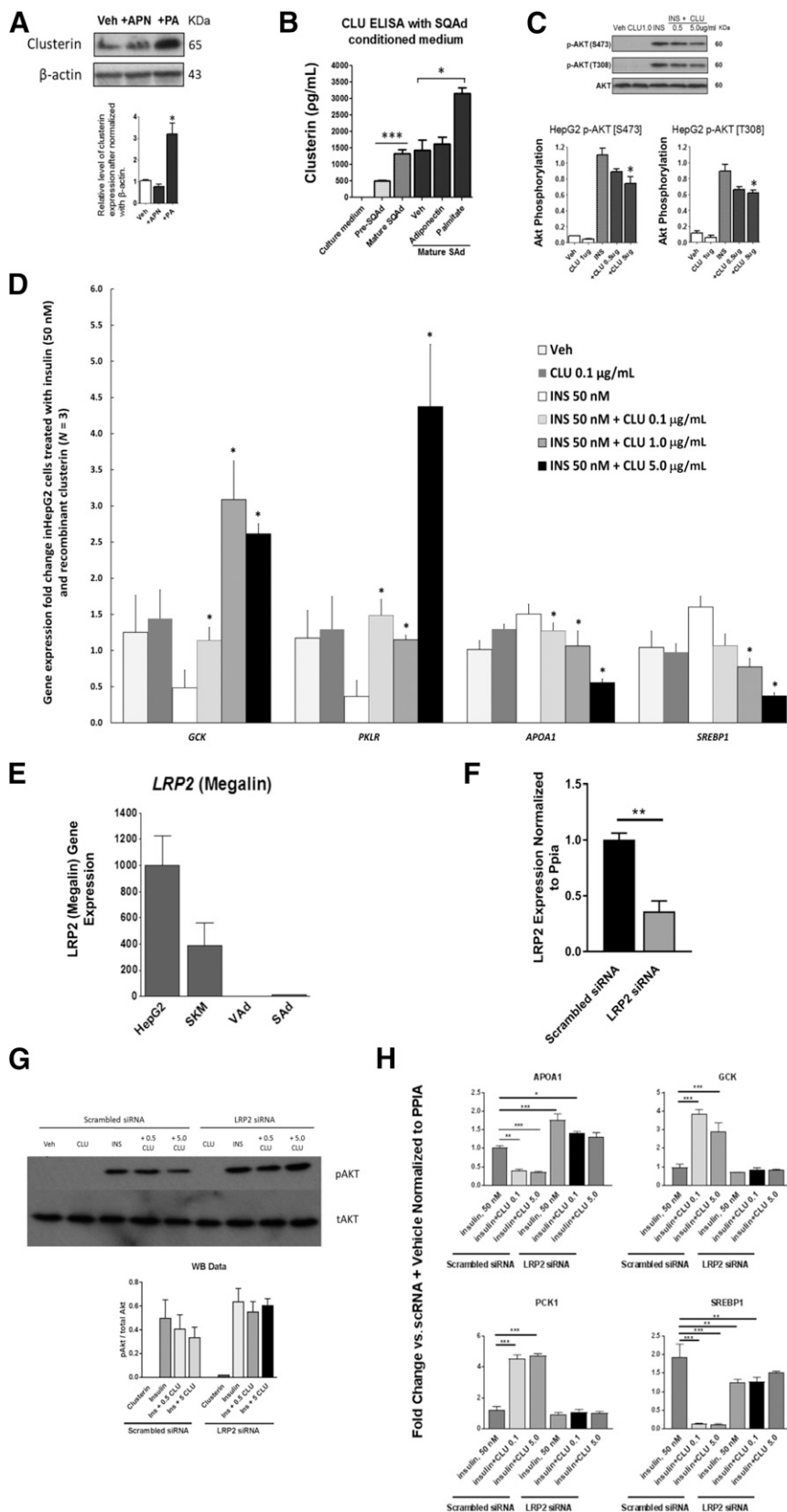


Figure 2—Clusterin protein in human subcutaneous (SQ) SAd treated with palmitate (PA) (200 μmol/L) and adiponectin (APN) (20 μg/mL) assessed by Western blotting of cell lysates (A) and ELISA (B) to determine the clusterin concentrations in conditioned media of the samples in A and the supernatant of the preadipocytes and mature (differentiated) adipocyte cultures without any treatment. C: Effect of recombinant clusterin (0.5 and 5.0 μg/mL) on insulin (INS)-stimulated protein kinase B (AKT) phosphorylation by Western blotting in HepG2 cells using two different phosphorylated AKT (p-Akt) antibodies. D: Effect of recombinant clusterin (0.1, 1.0, and 5.0 μg/mL) on insulin-stimulated expression by qRT-PCR of genes regulating

progressive increases in percent liver fat as measured by MRI (Fig. 3C), as well as histologic features of NASH, including ballooning degeneration, lobular inflammation, fibrosis, and NAS (Fig. 3H–J). In addition, we found positive correlations between adipocyte gene expression of clusterin and percent body fat and steatosis (Fig. 3F and G).

CONCLUSIONS

A strength of our investigation is the isolation and purification of human SAd, as multiple previous genomic analyses have used whole fat, which contains various mixtures of cells, making gene expression difficult to interpret. Our transcriptomics analysis of human SAd identified multiple ECM-related genes that differed between lean and obese subjects (6), but only SAd *CLU* (clusterin) was associated with systemic IR, multiple components of the metabolic syndrome (HDL cholesterol level, Tchol-to-HDL cholesterol ratio, TGs, SBP, and DBP), and overall CVD risk. Similarly, serum clusterin associated with several key lipid biomarkers and trended to associate with systemic IR. Supporting the gene expression findings, Western blotting demonstrated increased clusterin protein levels in obese compared with lean human adipocytes. Moreover, palmitate, a major circulating saturated fatty acid and component of a high-fat diet (34), enhanced clusterin expression and secretion in cultured human adipocytes. Recombinant clusterin attenuated insulin signaling in liver cells, dramatically increased genes involved in gluconeogenesis, and decreased expression of *SREBP1* and *APOA1*, providing several potential mechanisms by which clusterin could adversely affect systemic insulin sensitivity, promote hyperglycemia, and increase CVD risk. Thus, the liver is likely a major clusterin target, since clusterin did not affect insulin action in cultured human adipocytes or skeletal muscle cells. These hepatic effects appear to be mediated by *LRP2* (megalin). In

NASH-prone WD-fed aging *LDLR*^{-/-} mice, the progressive increase of adipocyte *CLU* expression paralleled the increase in liver fat, hepatic fibrosis, and steatohepatitis, suggesting an adipocyte-clusterin-liver relationship in both humans and mice. Our findings are consistent with previously observed elevations of serum clusterin in obese mice and humans and, yet, considerably expand upon these findings to delineate a unique role for adipocyte-derived clusterin in propagating the metabolic syndrome, IR, and CVD risk, likely through its hepatic effects.

Adipocyte *CLU* (Clusterin) Expression May Integrate Obesity, the Metabolic Syndrome, and Cardiovascular Risk

Recent studies indicate that adaptive and innate immune functions of the adipocyte regulate the overall immune cell composition of AT, which is progressively proinflammatory during the transition from a lean to obese state (35). In addition, as adipocytes expand, hypoxia stimulates excessive and pathologic ECM production (12). Within AT, a complex coordination between adipocytes and stromal cells, particularly ATMs (36), influences the AT ECM (37). Although both SAd and SATM express clusterin, SAd production appears to primarily influence systemic insulin action and the metabolic syndrome.

Clusterin (encoded by *CLU*) is a molecular chaperone that assists folding of secreted proteins (19,38) and facilitates the clearing of cellular debris and misfolded proteins from the ECM (39). In humans, there are two proteins encoded by the *CLU* gene: secretory *CLU* (s*CLU*) and nuclear *CLU* (n*CLU*) (40). Overexpression of s*CLU* protects cells from apoptosis, and a role for clusterin has previously been reported in oxidative stress-related diseases including various cancers (41) and Alzheimer disease (42). Its role in cardiometabolic disease has also recently emerged.

In the only study of AT *CLU*, a 2-week very-low-calorie diet reduced plasma clusterin levels, and whole fat clusterin

mRNA decreased after sleeve gastrectomy (43). In obese subjects, plasma clusterin levels were elevated and positively associated with BMI, inflammation (hs-CRP and retinol-binding protein-4) (16), and IR (14). Our results in human SAT considerably enhance these observations by demonstrating that adipocyte-specific *CLU* expression and protein levels are upregulated in human obesity, both *CLU* expression and serum clusterin positively correlate with IR, and the saturated fatty acid palmitate, known to induce IR and increased by a high-fat diet (44), is a potent stimulus for increased adipocyte *CLU* expression and secretion.

Most reports have confirmed an adverse effect of serum clusterin on CVD risk in humans. Plasma clusterin levels followed a graded increase with the number of metabolic syndrome components and associated positively with proinflammatory hs-CRP (16) but negatively with leptin in obesity-related CVD (45). In addition, clusterin had a deleterious effect on the antioxidant activity of paraoxonase-1, whose deficiency reportedly enhances atherosclerosis (46).

We describe herein that adipocyte expression of the gene *CLU* is directly related to multiple high-risk biomarkers of CVD (TGs, Tchol-to-HDL cholesterol ratio, SBP, and DBP), as well as heightened overall cardiovascular risk, and inversely correlated with HDL. All of these associations were independent of age, sex, smoking status, and BMI, indicating that adipocyte *CLU* expression may be a novel independent IR and CVD risk factor. Moreover, serum clusterin levels were an additional biomarker of higher TGs and Tchol-to-HDL cholesterol ratio and reduced HDL. *APOA1* is a major protein associated with HDL cholesterol particles in plasma that facilitates efflux of cholesterol from cells, notably from macrophages within atherosclerotic plaques, to the liver for excretion. Low plasma *APOA1* levels are also a strong predictor of CVD (29). Our finding of reduced hepatic

gluconeogenesis (*GCK* [glucokinase], *PKLR* [pyruvate kinase isozyme R], and *SREBP1*) and *APOA1* (apolipoprotein A1) in HepG2 cells. **P* < 0.05 vs. insulin samples by one-sample *t* test. All experiments were completed in triplicate. E: Relative gene expression of clusterin receptor LDL receptor-related protein 2 (*LRP2*) (megalin) in HepG2, cultured skeletal muscle cells (SKM), and VAd and SAd. F: *LRP2* expression in HepG2 cells treated with *LRP2* scRNA vs. siRNA and clusterin (2 ng/mL). G: Effect of recombinant clusterin on insulin-stimulated AKT phosphorylation (pAkt) by Western blotting in HepG2 cells transfected with control nontargeting scRNA and *LRP2*-targeted siRNA. Experiments were performed in duplicate; therefore, statistical analysis was not performed. tAkt, total AKT. H: Effect of recombinant clusterin on insulin-stimulated adipocyte gene expression of *LRP2*, *APOA1*, *GCK*, *PCK1*, and *SREBP1* by qRT-PCR in HepG2 cells transfected with nontargeted scRNA and *LRP2*-targeted siRNA. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Veh, vehicle.

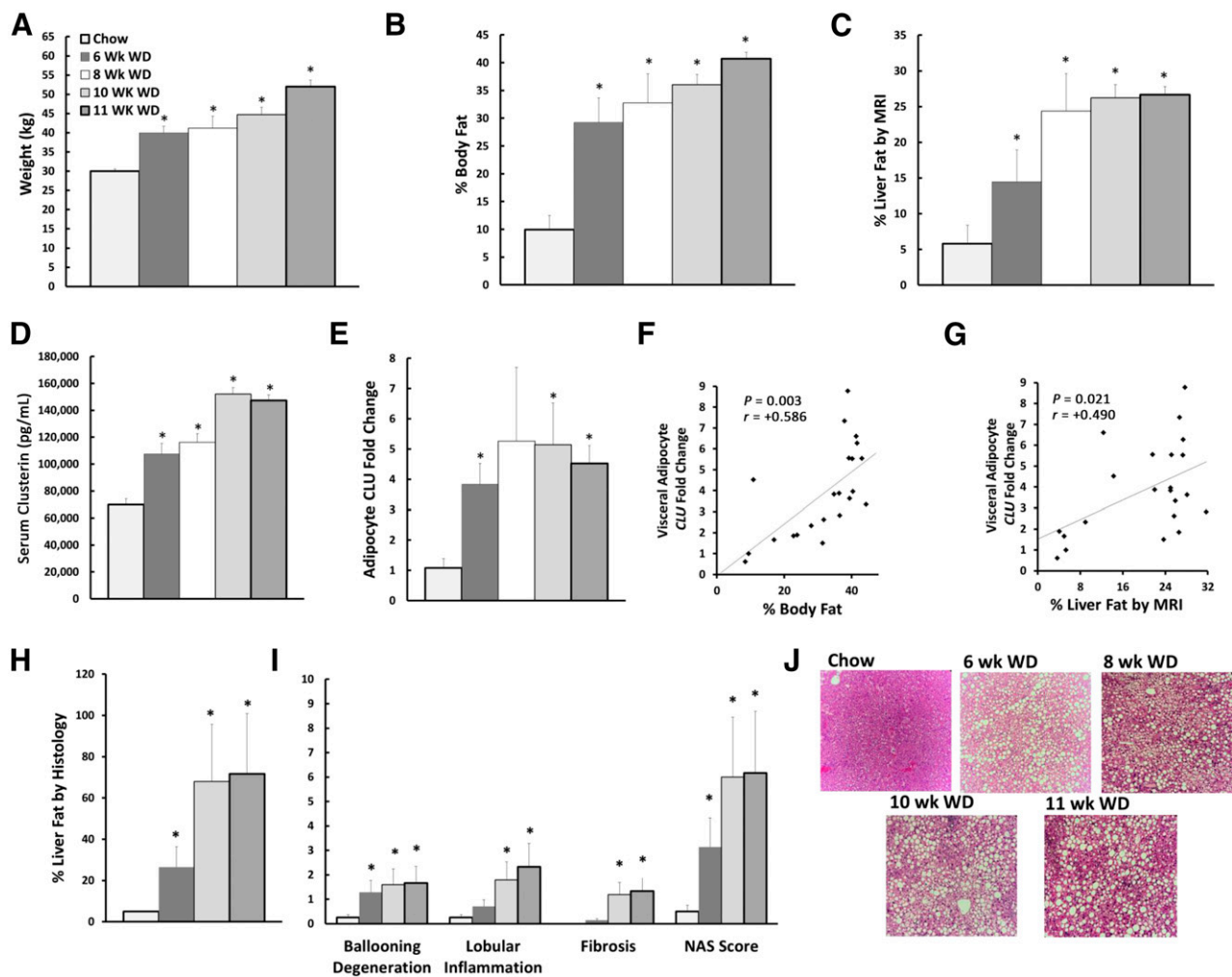


Figure 3—Weight (kg) (A), percent body fat (B), percent liver fat by MRI (C), serum clusterin (D), VAd *CLU* gene expression (E), percent liver fat by histology (H), and individual components of the NAS score (ballooning degeneration, lobular inflammation, and fibrosis) and total NAS score (I) in NASH-prone LDLR^{-/-} mice fed high-fat WD vs. normal chow ($n = 4$) for 6 ($n = 7$), 8 ($n = 5$), 10 ($n = 5$), and 11 ($n = 6$) weeks (wk). Correlation analyses (Spearman) between adipocyte gene expression of *CLU* by qRT-PCR vs. percent body fat (F) and percent liver fat (G) by MRI. J: Representative pathologic liver specimens in LDLR^{-/-} mice fed WD vs. normal chow for 6, 8, 10, and 11 weeks. * $P < 0.05$ vs. chow.

APOA1 expression induced by clusterin provides one potential mechanism for increased cardiovascular risk that necessitates further investigation of its impact on HDL cholesterol levels.

Clusterin Promotes Hepatic IR Through the LRP2 Receptor

Clusterin's effects on IR appear to be predominantly mediated in the liver. Addition of recombinant clusterin to human liver cells suppressed insulin signaling and promoted hepatic glucose production through upregulation of *GCK* and *PKLR* and downregulation of *SREBP1*, despite the presence of insulin. Knockdown of *SREBP1* perpetuates hyperglycemia via enhanced gluconeogenesis and reduced glycolysis/glycogen synthesis, even though it also limits hepatic lipogenesis (31). These insulin-

inhibiting effects were not seen in skeletal muscle cells or adipocytes. The liver actions are mediated by binding to LRP2, which we found to be highly and preferentially expressed in hepatocytes compared with skeletal muscle cells and adipocytes. The LRP2 protein is critical for the reuptake of numerous ligands (47) and acts to facilitate clusterin-potentiated lysosomal protein degradation, most prominently in the hypothalamus (21) and the neurovasculature (48). Yet, the role of hepatic clusterin binding to LRP2 is poorly defined.

Adipocyte *CLU* Expression Is Related to the Development of Hepatic Steatosis in Mice

We used an aged (65–74 weeks old) LDLR^{-/-} mouse model, which develops obesity-related hyperinsulinemia, IR,

hypertriglyceridemia, and NASH (27,33). We now show further resemblance to humans, since these mice also have increased adipocyte *CLU* expression, which is significantly associated with hepatic steatosis and parallels the time course of fibrosis and pathologic NASH scoring. We did not find a change in hepatic LRP2 expression with progression of disease (data not shown), and human liver samples were not available from the study participants. Nevertheless, these observations suggest that adipocyte *CLU* may be related to NASH development in mice; further studies in this model, particularly focused on liver effects of clusterin, will be useful. These results also have implications for CVD risk, since in humans there are multiple epidemiologic studies linking NASH to CVD (reviewed in [49]).

This study has several limitations. Despite no significant difference in age or BMI, there was a higher proportion of males than females among lean participants in our validation group, while sex among obese subjects was evenly distributed. Even after multivariate adjustment including sex, however, our findings still consistently demonstrated potentially important relationships between clusterin, cardiometabolic risk factors, and IR. In addition, there were no significant differences in serum clusterin or *CLU* expression based on sex. As a cross-sectional study, many of our findings are correlative and do not directly imply cause and effect. However, our data in human cultured cells treated with recombinant clusterin support a possible mechanistic role for clusterin in lipid and glucose homeostasis.

In conclusion, our study highlights that adipocyte-derived clusterin may be an important protein that integrates obesity with the metabolic syndrome and cardiovascular risk/mortality. A high-fat diet in both mice and humans increases palmitate, which stimulates adipocyte production of clusterin, leading to enhanced circulating levels. Clusterin binding to the hepatic LRP2 receptor then could inhibit insulin signaling to promote IR and gluconeogenesis; decrease APOA1 expression, leading to decreased HDL cholesterol; and enhance fatty liver/NASH. Further investigation is needed to determine whether adipocytes communicate with the liver through clusterin to instigate the metabolic syndrome; clusterin is one of the first ECM factors linking the enlarging adipocyte in human obesity to cardiometabolic disease.

Acknowledgments. The authors thank the study subjects for their participation.

Funding. This study was supported by American Diabetes Association grant 1-16-ICTS-049, National Institutes of Health KL2 Scholar Program award KL2TR001068 and grant HL135622, the John S. Dunn Research Foundation, and the Ting Tsung and Wei Fong Chao Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. D.B. and A.B. assisted in designing the study, collated and analyzed data, and wrote the manuscript. Z.Y., V.W., K.A., and S.T.C.W. assisted with the experiments, performed data analysis, contributed to the discussion, and reviewed/edited the manuscript. J.L. assisted with the experiments and reviewed/edited the manuscript. J.J.J. reviewed/edited the manuscript. B.N., S.N., and D.R. performed

the surgical biopsies and reviewed/edited the manuscript. M.Y. performed the histologic analysis and reviewed/edited the manuscript. S.T.C.W. additionally contributed to the design of the study. W.A.H. designed the study, obtained funding, reviewed the data, contributed to the discussion, and reviewed/edited the manuscript. D.B. and W.A.H. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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