Hepatic Notch Signaling Correlates With Insulin Resistance and Nonalcoholic Fatty Liver Disease

Luca Valenti,[1] Rosa M. Mendoza,[2] Raffaela Rametta,[1] Marco Maggioni,[3] Chris Kitajewski,[4] Carrie J. Shawber,[4] and Utpal B. Pajvani[2]

Hepatic Notch signaling is inappropriately activated in obese/insulin-resistant mouse models. Genetic or pharmacologic inhibition of hepatic Notch signaling in obese mice simultaneously improves glucose tolerance and reduces hepatic triglyceride content. As such, we predicted that Notch signaling in human liver would be positively associated with insulin resistance and hepatic steatosis. Here, we systematically survey Notch signaling in liver biopsy specimens, and show active Notch signaling in lean and obese adults, with expression of multiple Notch receptors and ligands. In morbidly obese patients undergoing bariatric surgery, we show that Notch activation positively correlates with glucose-6-phosphatase (*G6PC*) and phosphoenolpyruvate carboxykinase (*PCK1*) expression, key regulators of hepatic glucose output. We used immunofluorescence to identify active Notch signaling in hepatocytes and show highest activity in hyperglycemia, which we confirmed is a direct effect of hyperglycemia and insulin resistance. In a validation cohort of leaner individuals undergoing percutaneous liver biopsy for suspected nonalcoholic fatty liver disease (NAFLD), Notch activity showed independent positive association with insulin resistance and hepatic steatosis. Notably, Notch activity showed stronger correlation with the NAFLD activity score and alanine aminotransferase levels than with steatosis alone, suggesting that Notch activity is associated with nonalcoholic steatohepatitis. In summary, this study establishes that Notch signaling is activated in and may represent a therapeutic target for patients with obesity-related liver disease. *Diabetes* 62:4052–4062, 2013

O besity manifests as multiple pathologic states in the liver. Insulin resistance in adipocytes results in unrestrained lipolysis, with consequent excess free fatty acid flux to the liver (1). In a parallel pathogenic process, excess adiposity leads to insulin resistance, which begets the fasting hyperglycemia of type 2 diabetes (T2D) (2). Compensatory hyperinsulinemia drives de novo lipogenesis (3), and coupled with an impaired ability to catabolize and export fatty acids (4), results in excess hepatocyte triglyceride accumulation, or nonalcoholic fatty liver disease (NAFLD), in the presence of a predisposing genetic background (5). Steatosis may be associated with hepatocellular damage

and necroinflammatory changes, defining nonalcoholic steatohepatitis (NASH), which predisposes to cirrhosis and hepatocellular cancer (1). Interestingly, NASH further exacerbates hepatic insulin resistance through activation of forkhead box class O (FoxO) 1, the key transcriptional activator of glucose-6-phosphatase (*G6PC*) and phosphoenolpyruvate carboxykinase (*PCK1*), rate-limiting enzymes of gluconeogenesis and glycogenolysis, which combined regulate hepatic glucose output. This vicious cycle results in coincident NAFLD and T2D, which show independent associations with cardiovascular disease (6). No approved pharmacologic therapy is approved for NALFD, and although multiple T2D therapies are available, few show durability and long-term efficacy (7). As such, novel therapeutic directions are necessary to reduce overall obesity-related morbidity.

We previously showed that inhibition of hepatic Notch signaling protects from both obesity-induced glucose intolerance, by suppressing hepatic glucose output (8), and fatty liver, by reducing de novo lipogenesis (9). Notch signaling is highly conserved from lower organisms to primates and is critical for cell fate decision making, including regulation of cell specification and lineage restriction, depending on the cellular context (10). In mammals, cell surface Notch ligands of the Jagged (-1 and -2) and Delta-like (-1, -3, -4) families bind one of four Notch receptors (Notch1–4) on a neighboring cell, resulting in a series of cleavage events that culminate in the transcription of canonical Notch targets, the hairy enhancer of split (*HES*) and *Hes-related* (*HEY*) family of genes (11). Homozygous null alleles in this pathway result in embryonic lethality in mice (12–14) and loss-of-function mutations in severe developmental defects in affected individuals (15,16), proving the critical role of Notch signaling to regulate cell fate decisions in normal development.

Less is known about Notch signaling in mature tissue. Increased Notch expression and function has been shown in cancer and tumor angiogenesis (17–19), but there are few reports of expression analysis of Notch pathway proteins in developed, non-neoplastic tissue. In the liver, Notch proteins are constitutively expressed in multiple cell types (20,21), with increased expression in hepatocytes after partial hepatectomy (22). In rat models, normal liver regeneration was prevented by genetic inhibition of hepatocyte Notch signaling (23). As such, we predicted that the Notch-signaling apparatus is present in mature liver and may be induced by obesity-induced hepatocyte damage. Our initial characterization of Notch signaling in murine liver demonstrated that Notch target gene expression is, in fact, increased in mouse models of obesity and insulin resistance (8). Interestingly, genetic (8) or pharmacologic (11,24,25) blockade of hepatocyte Notch signaling resulted in parallel inhibition of hepatic glucose production (8) as well as triglyceride accumulation (9), lowering the overall atherosclerotic burden in obese mice (26).

Conversely, constitutive activation of hepatocyte Notch signaling caused glucose intolerance and fatty liver (8,9). These studies suggested that the Notch pathway is active through adulthood in rodent liver, is inappropriately stimulated by obesity, and may be manipulated to reduce the obesity-related metabolic disease burden.

On the basis of these rodent studies, we hypothesized that Notch signaling is similarly functional and may correlate with disease severity in patients with hepatic insulin resistance and NAFLD. In this study, we show that Notch proteins and ligands are expressed in lean and obese subjects and that increased activation of this pathway, as assessed by expression of Notch target genes of the *HES/HEY* family, positively correlates with gluconeogenic gene expression and hyperglycemia in a cohort of morbidly obese patients undergoing bariatric surgery. In a validation cohort across a range of BMIs, we confirmed the positive association between *HES/HEY* family genes and insulin resistance as well as demonstrated an independent positive association with hepatic fat content. Finally, we show that hepatic Notch signal activation correlates better with measures of liver inflammation than with simple steatosis (SS), suggesting that it may represent a marker of the transition from SS to NASH. This work establishes that Notch signaling correlates and potentially represents a novel therapeutic target for both arms of obesity-related liver disease, T2D and NAFLD.

RESEARCH DESIGN AND METHODS

Subjects. The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board and ethical committee of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda. Each subject gave written informed consent. Demographic and anthropometric features, arterial blood pressure, medical history, and medications were recorded for all patients and are summarized in Table 1. A needle liver biopsy was performed in all patients. The specimen was formalin-preserved for histological and immunofluorescence analysis. Part of the sample was included in RNAlater (Ambion, Carlsbad, CA), immediately frozen in liquid nitrogen, and stored at −80°C for RNA analysis.

Bariatric surgery clinic. We recruited 44 of 48 consecutive patients who underwent bariatric surgery at the IRCCS Ca' Granda Ospedale Policlinico di Milano, between 2006 and 2008. Indications for bariatric surgery included BMI >40 kg/m^2 or BMI >35 kg/m^2 in the presence of metabolic complications (T2D, uncontrolled hypertension, severe dyslipidemia, obstructive sleep apnea). We excluded subjects with alcohol consumption >30 g/day for men and 20 g/day for women ($n = 1$) and chronic viral hepatitis ($n = 3$). Fasting glucose, HDL and total cholesterol, triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were assessed the day of surgery, and needle liver biopsy (16-gauge) was performed during the bariatric surgery. Insulin-resistance status was classified according to fasting glucose levels and oral glucose tolerance test results. These patients are part of a previously reported cohort in which we characterized insulin-dependent signaling and the regulation of lipid metabolism according to liver histology (27).

Hepatology clinic patients. We recruited 38 unrelated patients followed up at the Metabolic Liver Diseases outpatient service, Fondazione IRCCS Ca' Granda, who underwent percutaneous liver biopsy because of suspected NAFLD due to persistently abnormal liver enzymes/serum ferritin test results or a history of steatosis associated with severe metabolic abnormalities, between January 2011 and January 2012. Other causes of liver disease were excluded, including increased alcohol intake (>30 g/day for men or 20 g/day for women), viral and autoimmune hepatitis, hereditary hemochromatosis, and α1-antitrypsin deficiency. Fasting glucose and insulin levels, HDL and total cholesterol, triglycerides, and ALT and AST levels were assessed the day of the biopsy. Patients were classified as insulin-sensitive based on homeostasis model assessment–insulin resistance (HOMA-IR) <2.5 ([fasting insulin (μU/mL) × fasting glucose (mmol/L)]/22.5) (28) without a history of impaired glucose tolerance or impaired fasting glucose, insulin resistance (HOMA-IR >2.5, or impaired fasting glucose or impaired glucose tolerance, but no diagnosis of diabetes) or T2D.

Histological analysis. A single expert pathologist, unaware of gene expression and immunofluorescence data, evaluated all biopsy specimens according to Kleiner et al. (29), based on the determination of the NAFLD activity score (NAS) as the result of the sum of steatosis severity (0–3), intralobular necroinflammation (0–3), and hepatocellular ballooning (0–2). Steatosis percentage was determined in at least 10 hepatic lobules per patient. Subjects were classified in three groups according to liver histology: histologically normal liver, SS, and NASH.

Immunofluorescence. Paraffinized sections were deparaffinized, rehydrated, and stained as previously described (30). To determine Notch signal activation, tissues were stained with Hey1 (#5714, 1:100 dilution) or HeyL (#10094, 1:150 dilution) antibodies from Millipore and detected with donkey anti-rabbit Alexa Fluor 488 (1:1,000 dilution) or donkey anti-mouse Alexa Fluor 594 (1:1,000) from Invitrogen. Slides were mounted with Vectashield with DAPI (Vector

TABLE 1
Demographic and clinical features of subjects included in the study subdivided according to the case series (bariatric surgery and hepatology clinic) and liver histology

	Bariatric surgery				Hepatology clinic			
	Normal	SS	NASH	P value	Normal	SS	NASH	P value
n (%)	5 (12)	14 (33)	23 (55)		7 (18)	11 (29)	20 (53)	
Sex (n)				0.035				0.23
Female	5	13	14		0	1	0	
Male	0	1	9		7	10	20	
Age (years)	44 ± 4	40 ± 9	42 ± 10	0.73	45 ± 12	54 ± 9	51 ± 12	0.33
BMI (kg/m^2)	40.8 ± 10	40.5 ± 10	43.5 ± 8	0.54	25.1 ± 2.4	27.5 ± 3.4	28.7 ± 4.2	0.11
Abdominal circumference (cm)	—	—	—	—	94 ± 6	102 ± 6	112 ± 21	0.05
Glucose (mg/dL)	83 ± 8	88 ± 5	117 ± 56	0.09	92 ± 7	116 ± 35	111 ± 30	0.24
Insulin (IU/mL)	—	—	—	—	8.4 ± 2.2	14.1 ± 1.9	16.0 ± 1.5	0.034
HOMA-IR	—	—	—	—	1.7 ± 0.4	3.6 ± 1.7	3.8 ± 1.7	0.018
Glucose tolerance				—				0.001
IS [n (%)]	—	—	—		7 (100)	2 (18)	4 (20)	
IR [n (%)]	—	—	—		0	5 (46)	10 (50)	
T2D [n (%)]	0	0	9 (39)		0	4 (36)	6 (30)	
Cholesterol (mg/dL)	215 ± 31	211 ± 36	214 ± 41	0.96	191 ± 47	201 ± 37	188 ± 40	0.66
Triglyceride (mg/dL)	99 ± 54	155 ± 95	157 ± 54	0.30	94 ± 27	98 ± 48	115 ± 54	0.51
HDL (mg/dL)	73 ± 11	52 ± 9	46 ± 12	<0.0001	55 ± 15	57 ± 28	44 ± 14	0.17
Steatosis (%)	2 ± 1	17 ± 13	61 ± 21	<0.0001	3 ± 2	19 ± 14	52 ± 23	<0.0001
ALT (IU/mL)	18 ± 5	26 ± 9	39 ± 25	0.03	22 ± 8	40 ± 32	66 ± 46	0.02

Data represent average ± SD unless indicated otherwise. IR, insulin-resistant; IS, insulin-sensitive.

Laboratories). Images were captured with a Nikon ECLIPSE E800 microscope and a Nikon DXM 1200 digital camera and processed with Image ProPlus software. Staining was quantitated in a blinded fashion from 1 (low expression) to 4 (high expression), independently by three investigators, and scores were averaged.

Quantitative RT-PCR. We isolated RNA with Trizol (Invitrogen), synthesized cDNA with Superscript III RT (Invitrogen), and performed quantitative PCR with a DNA Engine Opticon 2 System (Bio-Rad) and GoTaq SYBR Green (Promega). Absolute mRNA levels were determine for each gene using species- and primer-specific standard curves, then normalized to *18S*, and are presented as relative transcript levels (fg/ng *18S*). Primer sequences are available upon request.

Luciferase assays. Hepa1c1c7 cells were transfected with the Rbp-Jk reporter luciferase construct, as previously described (8), and then incubated in serum-free medium with variable glucose content in the presence or absence of 10 nmol/L insulin or 10 nmol/L glucagon.

Hepatocyte studies. We isolated and cultured primary mouse hepatocytes as described (8) and obtained human primary hepatocytes from Invitrogen. For gene and protein expression studies, we treated hepatocytes with variable concentrations of glucose, and/or 10 nmol/L insulin (Sigma-Aldrich) for 3 h, with all experiments completed by 24 h after isolation.

Statistical analysis. Results are shown as mean $\pm$ SEM. ANOVA was used for comparison of means among groups. Gene expression levels were correlated by the Pearson correlation. The association between *HES1* expression (above the median) and insulin resistance of T2D was evaluated by multivariate logistic regression analysis adjusted for age, steatosis, and BMI. Independent predictors of *HES1* expression were evaluated at multivariate regression analysis (generalized linear model), including NAS and insulin levels, the variables most significantly associated at univariate analysis. Differences were considered significant at $P < 0.05$ (two-tailed).

RESULTS

Human liver has a functional, evolutionarily conserved Notch-signaling apparatus. To clarify the role of Notch signaling in the postdevelopment human liver, we characterized liver Notch and ligand expression in insulin-sensitive subjects with a histologically normal liver. We found that in these subjects, all four Notch receptors were expressed, with relative higher levels of *NOTCH1* and *NOTCH2* (Fig. 1A, left panel). In addition, all five Notch ligands were detectable by quantitative PCR, with *JAG1* and *JAG2* showing greater expression than Delta-like ligands. Of note, this expression pattern is broadly similar to the distribution observed in murine liver (Fig. 1A, right panel) as well as in mouse hepatocytes (8). Generally, expression of *NOTCH1* and other Notch genes covaried (Fig. 1B) as well as with *HES/HEY* family target genes (Fig. 1C), but showed no correlation with housekeeping genes (*ACTB*, *GAPDH*) and a nonsignificant trend toward a negative correlation with Notch ligand expression (data not shown). These data show that Notch signaling components are present in mature liver and that relative expression is evolutionarily conserved.

Hepatic Notch signaling correlates with *G6PC/PCK1* expression and is increased in patients with T2D. Because Notch proteins and ligands are expressed in human liver, we hypothesized that Notch signaling may be
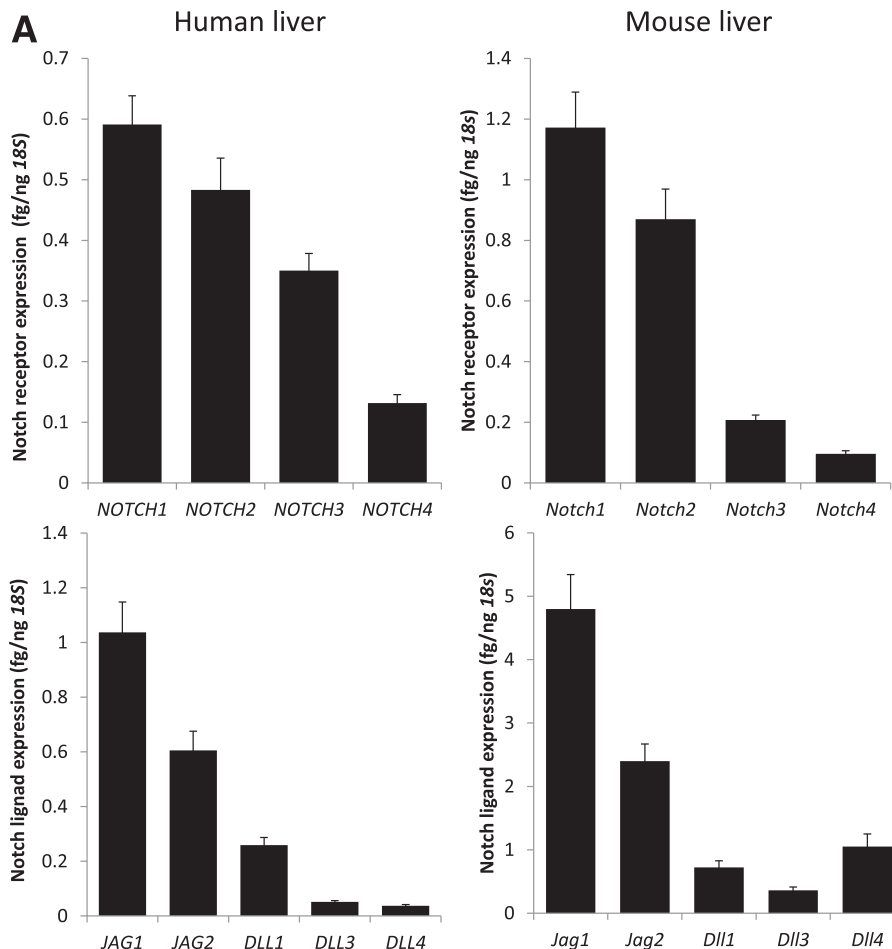

FIG. 1. Notch pathway in human liver. *A*: Notch receptor and ligand expression is similar in human (*left*) and mouse liver (*right*). Hepatic *NOTCH1* expression positively correlates with the expression of other Notch receptors ($P < 0.001$ by ANOVA for all comparisons) (*B*) and canonical Notch target *HES1* ($P < 0.001$ by ANOVA for all comparisons) (*C*). Data show means $\pm$ SEM. AU, arbitrary units.
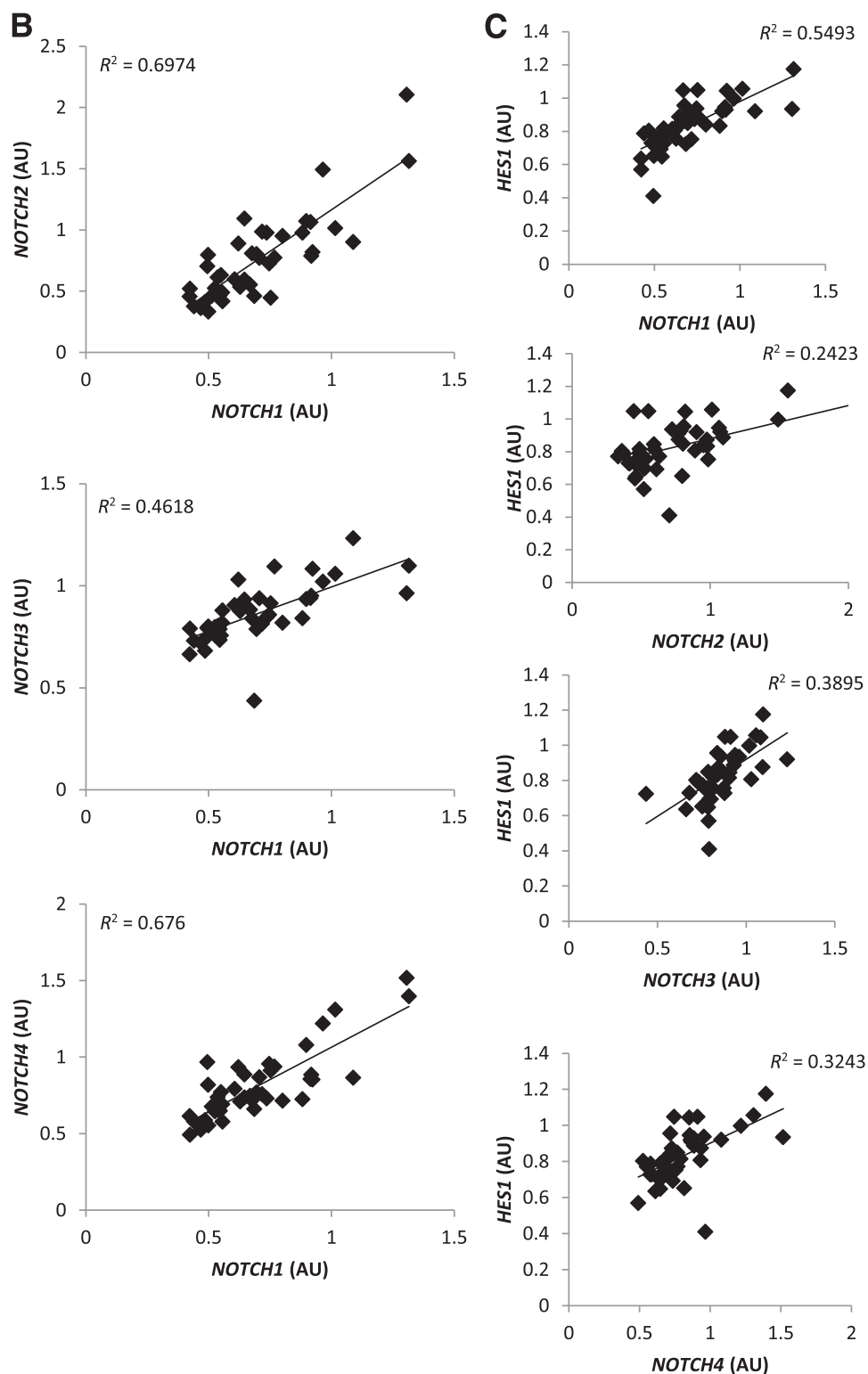
FIG. 1. Continued.

increased in obesity and insulin resistance as in mouse models. We performed a pilot study in 42 morbidly obese patients undergoing liver biopsy at time of gastric banding. Full demographic and clinical information is summarized in Table 1, but most of the subjects were female (76%), with SS or NASH (88%), and nondiabetic (79%). Expression of canonical Notch target genes of the *HES/HEY*

family positively correlated with hepatic expression of *G6PC* and *PCK1* (Table 2 and Fig. 2*A*). Of note, we saw no correlation in severely obese patients between Notch signaling and age, sex, or BMI (data not shown), which suggests that the correlations between *HES/HEY* and *G6PC/PCK1* were independent of overall adiposity, but correlations persisted or even strengthened when

TABLE 2
Correlation of Notch gene targets with *G6PC* and *PCK1* expression in 42 patients undergoing bariatric surgery

| | Overall series | | | | Patients without diabetes | | | |
| | *G6PC* | | *PCK1* | | *G6PC* | | *PCK1* | |
Gene	ρ	Pearson	ρ	Pearson	ρ	Pearson	ρ	Pearson
HES1	+0.39	0.011	+0.47	0.0016	+0.44	0.006	+0.35	0.03
HES6	+0.24	NS	+0.33	0.018	+0.35	0.03	+0.46	0.003
HES7	+0.27	NS	+0.35	0.033	+0.51	0.007	+0.45	0.008
HEY1	+0.27	NS	+0.42	0.002	+0.44	0.006	+0.41	0.01

diabetic patients were excluded from the analysis (Table 2).

Liver consists primarily of hepatocytes, but also non-hepatocyte residents, including endothelial, phagocytic Kupffer, and stellate cells (31). The relative contribution of hepatocytes to overall hepatic Notch signaling was unclear from gene expression studies from the whole liver, so we performed immunofluorescence for Notch targets on a representative subset of patients across a range of glycemic control. We observed predominantly hepatocyte staining of Notch targets HEY1 and HEYL (Fig. 2*B*). HEY1 hepatocyte staining and *HEY1* gene expression was very well correlated (Fig. 2*C*), as was HEYL staining and *HEYL*

expression (not shown), suggesting hepatic gene expression is a good surrogate for hepatocyte protein levels. Further, *HEY1* gene expression strongly correlated with *HES1*, *HEY1*, and other Notch targets (Fig. 2*D* and data not shown), allowing HEY1 staining as a surrogate for global hepatocyte Notch activation. Having validated the technique, we next examined sections from age- and BMI-matched normoglycemic and diabetic patients and noted a marked increase in HEY1 and HEYL staining in hyperglycemic patients (Fig. 2*E* and *F* and data not shown). These data establish that Notch signaling is present in adult hepatocytes and increased in T2D.
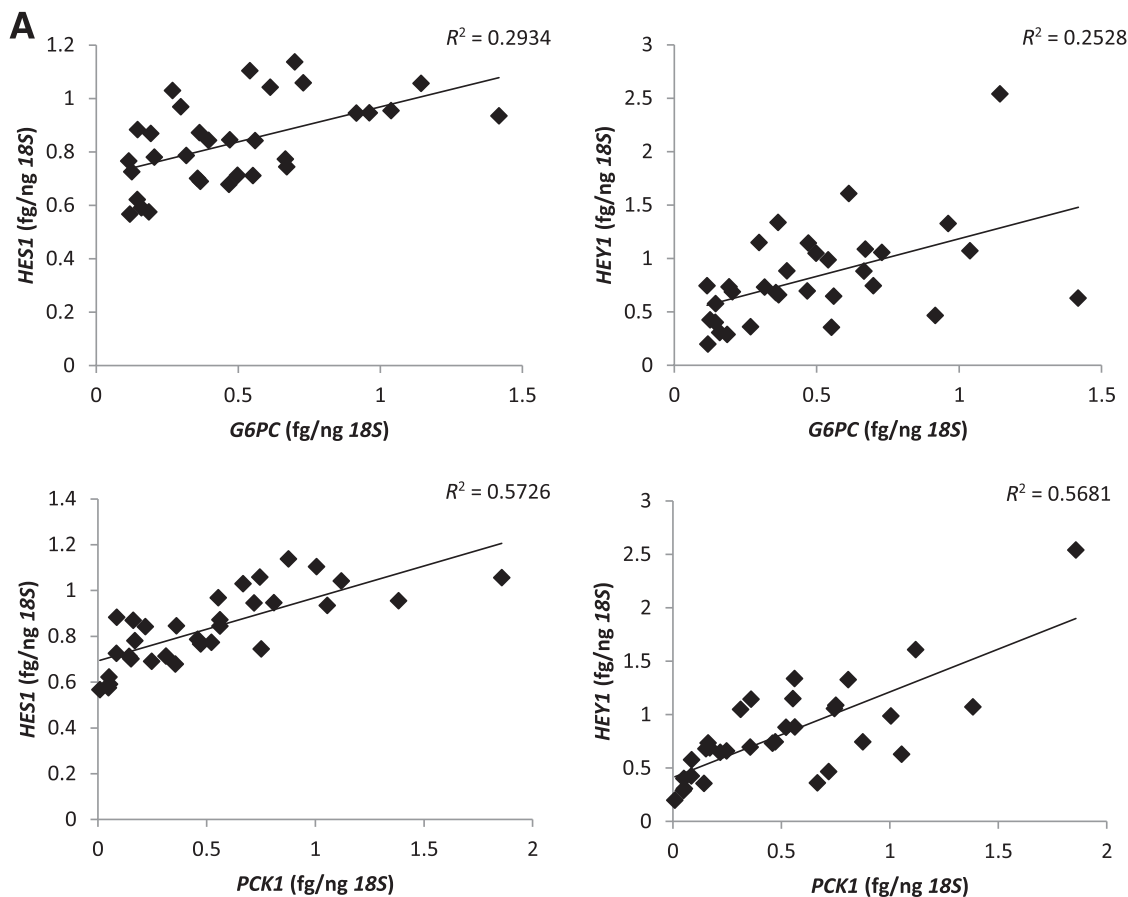

FIG. 2. Notch activity in liver increases with hyperglycemia. *A*: Correlation of Notch targets of the *HES/HEY* family with the expression of genes controlling hepatic glucose production, *G6PC* and *PCK1*. *B*: Representative liver sections, stained with antibodies to HEY1 (green, *left*) or HEYL (red, *middle*) and counterstained with DAPI (blue), with merged image (*right*). Hepatic *HEY1* expression correlates with Hey1 staining ($P < 0.001$ by ANOVA) (*C*) and *HES1* expression ($P < 0.001$ by ANOVA) (*D*). Immunofluorescence (*E*) and quantitation (*F*) of staining for HEY1 in formalin-fixed liver sections from non-T2D and T2D patients undergoing liver biopsy during gastric bypass surgery. The very bright spots are auto-fluorescent erythrocytes and are not included in the quantitation. Data show means ± SEM. *$P < 0.05$ vs. non-T2D patients. AU, arbitrary units.
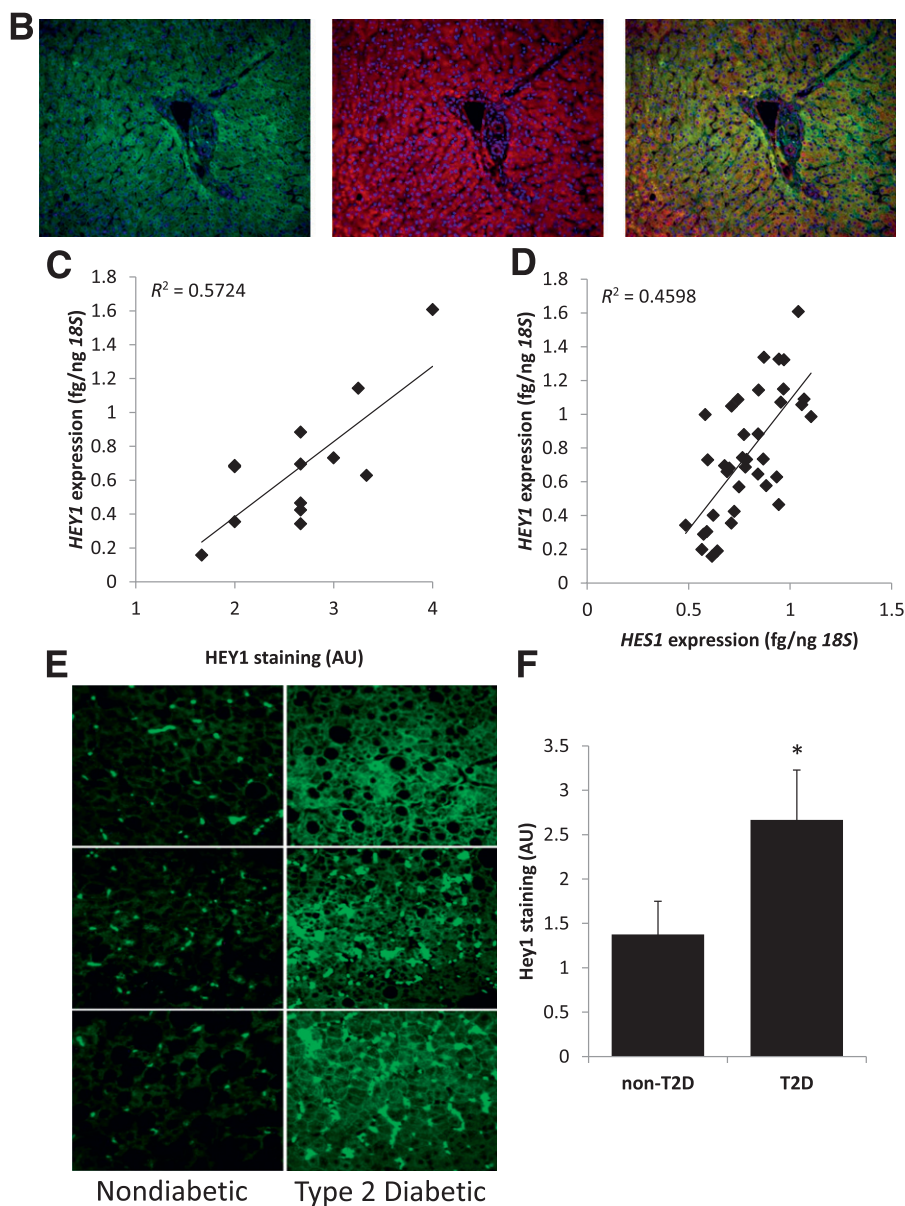
FIG. 2. Continued.

Hepatic Notch signaling is increased in insulin resistance. The positive correlation of *HES/HEY* family genes with *G6PC/PCK1* in obese patients (Table 2), coupled with increased HEY1 staining in hyperglycemia, suggested that Notch signaling is increased in insulin-resistant liver. To determine whether hepatic Notch signaling was similarly induced in leaner patients and might precede development of frank diabetes in insulin

TABLE 3
HES1 expression correlates independently with HOMA-IR as well as NAS scores

	Correlation coefficient	SE	Unadjusted P value*	Adjusted P value**
Age (per 10 years)	0.03	0.05	NS	NA
BMI (kg/m^2)	0.01	0.02	NS	NA
Glucose (per 10 mg/dL increase)	0.00	0.02	NS	NA
Insulin	0.02	0.01	0.002	0.03
ALT (per 10 IU/L increase)	0.07	0.02	0.003	NA
HOMA-IR	0.11	0.03	0.002	NA
% Steatosis (per 10% increase)	0.05	0.02	0.011	NA
NAS	0.11	0.03	<0.0001	0.03

NA, not addressed. *At univariate analysis (generalized linear model). **At multivariate analysis (generalized linear model) including insulin levels and NAS score, the strongest variables related to insulin resistance and histological damage, respectively, at univariate analysis.
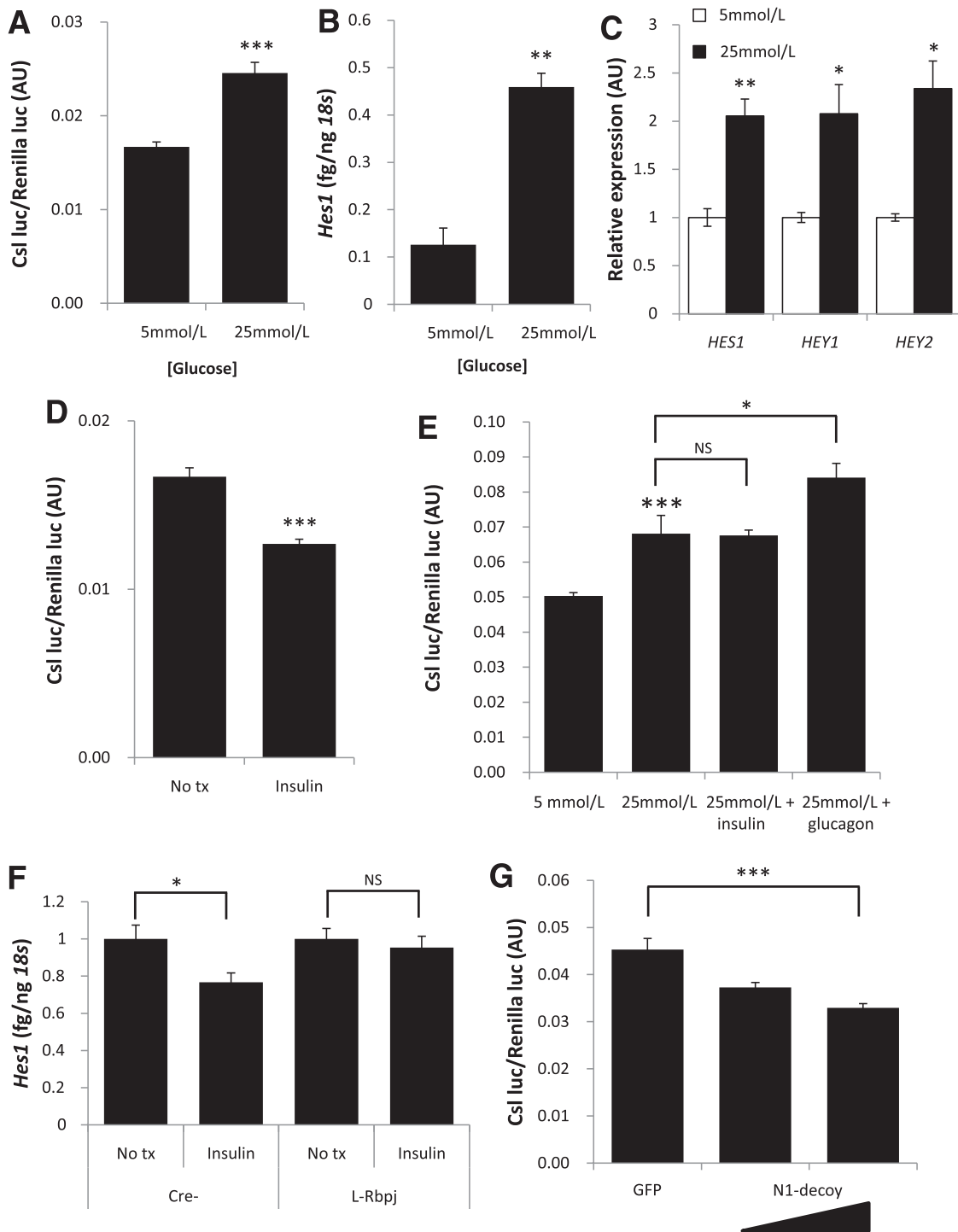
FIG. 3. Notch activity in hepatocytes increases with hyperglycemia. Notch-luciferase reporter (Csl-luc) expression in Hepa1c1c7 hepatoma cells (*A*), and Notch target gene expression in primary hepatocytes from mice (*B*) or human donors (*C*) is increased with transient exposure to hyperglycemic (25 mmol/L glucose) conditions. Insulin reduces Notch activation in Hepa1c1c7 hepatoma cells when cultured in normoglycemic (*D*), but not hyperglycemic conditions (*E*), even as glucagon has a synergistic effect. *F*: Insulin fails to repress *Hes1* expression in hepatocytes derived from mice lacking hepatic Notch signaling. *G*: Hyperglycemic-induced Notch reporter expression in Hepa1c1c7 hepatoma cells is abrogated by transduction with N1-decoy, which blocks ligand-dependent Notch signaling. Data show means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. 5 mmol/L glucose or control (Cre- or GFP-transduced) cells. AU, arbitrary units.

resistance, we analyzed specimens from 38 consecutive outpatients undergoing percutaneous liver biopsy. Full demographics can be found in Table 1, but this group was 97% male, with an average BMI in the overweight range (25–30 kg/m^2). In these patients, we again noted that *HES/HEY* family genes correlated with *G6PC* and *PCK1*, as well

as with each other (data not shown), suggesting that Notch and gluconeogenic gene expression coregulation is not specific to obese patients. As in the bariatric surgery cohort, Notch signaling did not vary by age, BMI, or abdominal circumference, but as predicted, we found a significant positive correlation with Notch targets (*HES1*) and plasma
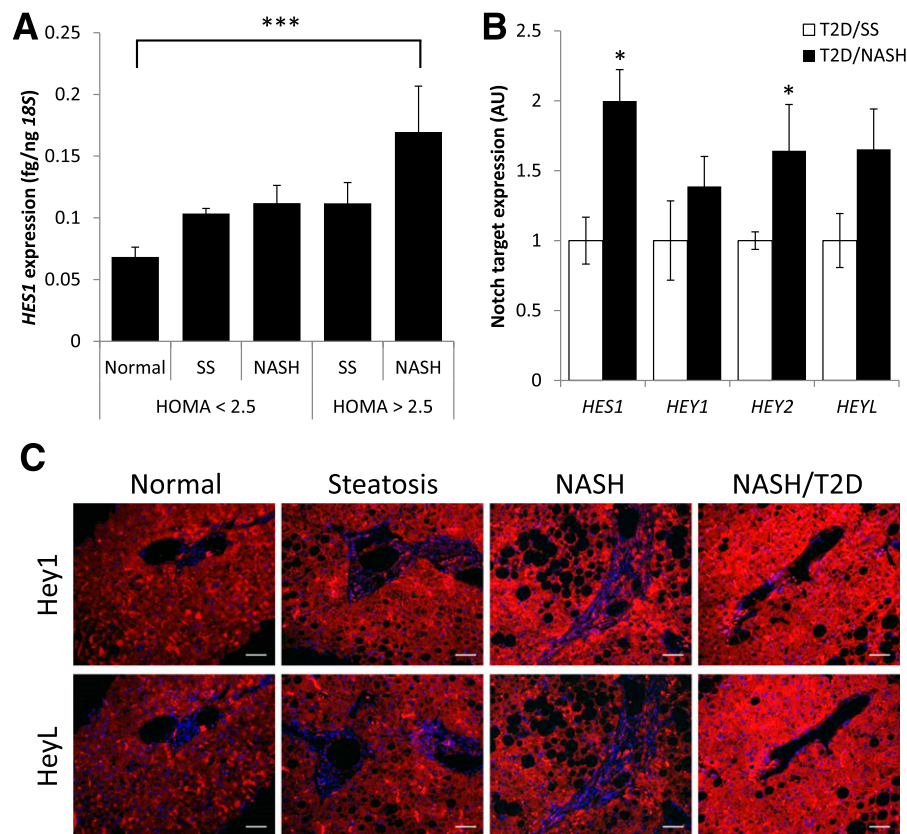
FIG. 4. Notch-dependent gene expression is progressively increased in insulin resistance and NAFLD severity. Quantitative PCR for *HES1* and other Notch target genes from liver biopsy samples from patients with pathologically confirmed SS, NASH, or normal liver, further subdivided as insulin-sensitive (HOMA-IR <2.5) vs. insulin-resistant (HOMA-IR >2.5) (*A*), or T2D patients (*B*), are shown. *C*: Hepatocyte Notch target expression is increased in patients with NASH. Scale bars are 50 μm long. Data show means ± SEM. *P < 0.05 vs. T2D/SS; ***P < 0.001 by ANOVA. AU, arbitrary units.

insulin levels (not shown) and HOMA-IR, independent of confounding factors (Table 3), which was not mitigated by exclusion of diabetic patients (data not shown). Multivariate logistic regression analysis showed increased *HES1* expression was positively associated with the presence of insulin resistance of diabetes (odds ratio [OR] 2.11 [95% CI 1.5–38]), together with age (OR 1.17 [95% CI 1.05–1.40]) and steatosis (OR 5.5 [95% CI 1.3–53]), independently of BMI. These data establish that Notch activation is found in the insulin-resistant liver, preceding frank hyperglycemia and the development of diabetes.

Insulin and hyperglycemia directly and reciprocally affect Notch signaling. To test the hypothesis that the apparent regulation of hepatic Notch signaling by insulin resistance and hyperglycemia is direct and cell-autonomous, we transfected hepatoma cells with a Notch-reporter luciferase construct and found increased Notch activation in hyperglycemic as opposed to basal conditions (Fig. 3A). Similarly, endogenous Notch target expression was higher in primary mouse or human hepatocytes transiently exposed to hyperglycemia (Fig. 3B and C). Insulin treatment had the opposite effect on Notch signaling, with insulin-treated cells showing decreased reporter activation (Fig. 3D). Interestingly, the inhibitory effect of insulin, but not a synergistic effect of glucagon, was lost in cells chronically cultured in hyperglycemic conditions (Fig. 3E), Further, insulin was no longer able to repress *Hes1* expression in primary hepatocytes derived from mice lacking Rbp-Jk (8), the common transcriptional effector of Notch1–4 signaling (Fig. 3F). Similarly, pharmacologic application

of a novel Notch antagonist, an ectodomain "decoy" that quenches ligand-dependent Notch signaling (24), abrogated hyperglycemia-induced Notch-reporter activity (Fig. 3G). In sum, these data suggest a cell-autonomous, dynamic regulation of hepatic Notch signaling by metabolic stimuli.

Hepatic Notch signaling is positively correlated with hepatic steatosis and inflammation. Beyond the positive correlation between *HES/HEY* gene expression and measures of insulin resistance and hyperglycemia, we predicted that Notch signaling would correlate with hepatic lipid content and markers of necroinflammation. Indeed, when patients are subdivided by liver histology, we find that *HES1* and other Notch target genes are strongly upregulated across the spectrum from normal liver to SS to NASH, and further still in insulin resistance (Fig. 4A) or in patients with T2D (Fig. 4B). Notch target staining was similarly increased in hepatocytes of NASH patients compared with SS patients (Fig. 4C).

In fact, *HES1* expression more closely correlated to measures of hepatic inflammation than to steatosis, with stronger coefficients of correlation with NAS and ALT levels than with the percentage of steatosis (Fig. 5A–C and Table 3). As such, patients with a NAS score of 0–2, correlating with a low risk of NASH (29,32), had lower Notch, ligand, and *HES/HEY* family gene expression than those with scores of 3 or higher (Fig. 5D–F). Furthermore, the NAS score was associated with HES1 expression independently of insulin resistance (Table 3). In sum, these data suggest that hepatic Notch signaling is elevated in insulin resistance as well as in NASH.
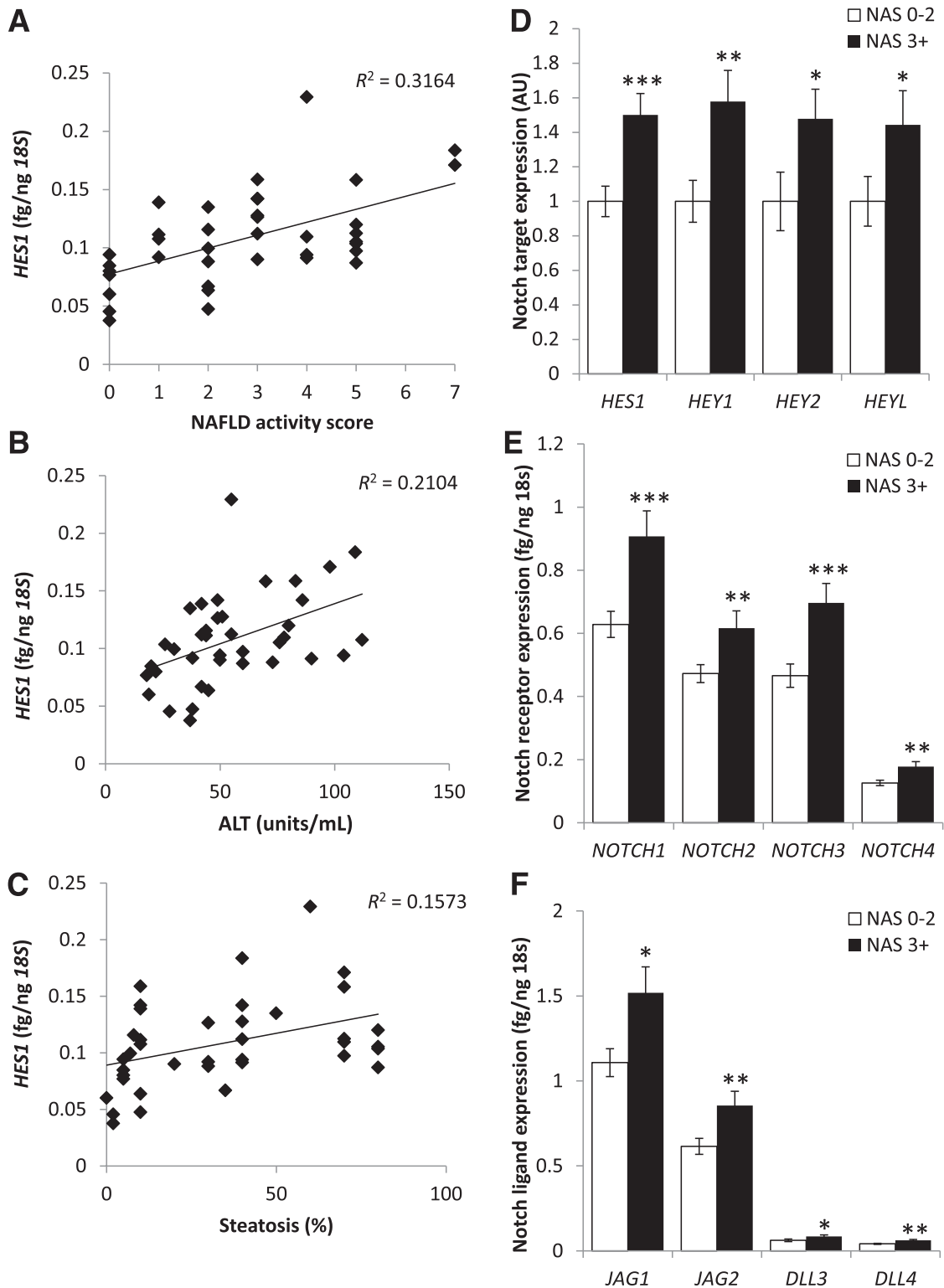
FIG. 5. Notch activity correlates with hepatocyte necroinflammation. Liver *HES1* expression was plotted against NAS (*A*), serum ALT level (*B*), and percentage of hepatic steatosis in patients undergoing percutaneous liver biopsy (*C*). Notch target (*D*), protein (*E*), and ligand gene expression (*F*) are increased in liver from patients with higher NAS (NAS 3+) compared with patients at low risk for steatohepatitis (NAS 0–2). Data show means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. NAS 0–2. AU, arbitrary units.

DISCUSSION

Notch signaling has been extensively studied in the context of differentiation or cancer (10), but its metabolic functions are novel. Rodent studies have demonstrated a postdevelopment role for Notch in the regulation of

obesity-induced insulin resistance/diabetes (8,26) and in liver fat accumulation (9), but whether this would translate to human disease was unclear. This work answers two important questions: hepatic Notch signaling is *1*) present in human liver/hepatocytes, and *2*) its activation is tied

to the metabolic state of the organism, with an independent positive correlation with measures of insulin resistance and hepatic steatosis/inflammation. Notch signaling appears to be inappropriately reactivated in the insulin-resistant liver, reprising its developmental role and reassociating with its molecular partners from differentiation that drives the metabolic effects of Notch signaling. For instance, the Notch transcriptional effector Rbp-Jk binds to and activates FoxO1 (33), a key transcriptional activator of hormone-stimulated hepatic glucose production (2,34), increasing functional hepatic insulin resistance (8). Similarly, Notch signaling activates the nutrient-sensitive mTorc1 pathway in the liver, increasing de novo lipogenesis and hepatic triglyceride (9). Mouse models with reduced hepatic glucose production often have compensatory fatty liver due to reoriented carbon flux; for instance, FoxO-knockout mice (35,36) or mice treated with glucokinase activators (37). Reduced Notch action allows the dissociation of insulin-signaling pathways in the liver, redressing insulin resistance without causing undue nutrient sensitivity, allowing for this rare dual therapeutic benefit independent of effects on body weight or adiposity.

It is intriguing that Notch activation more strongly correlates with markers of steatohepatitis, including the NAS and ALT levels, than with steatosis itself. With better imaging techniques (38), clinicians can increasingly diagnose excess hepatic fat (5). NAFLD is extraordinarily common, with a prevalence approaching 30% in many populations (39), leaving a therapeutic dilemma because only a subset of patients progress to NASH (40). As such, increased Notch signaling may represent a biomarker or even a causative factor for progression from SS to NASH. It is noteworthy that Notch pathway activation has similarly been found in human hepatocellular carcinoma (41) and causes hepatic fibrosis and tumor formation in mice (41–43), which potentially explains the predisposition of patients with NASH to develop cirrhosis and hepatocellular carcinoma (44,45).

One of the major limitations of this type of observational study is that we are able to detect Notch signaling, as well as markers of insulin sensitivity and hepatic fat, at one moment in time. Longitudinal studies (46) are required to determine whether altered Notch signaling predates or predicts the development of worsening steatosis or progression to NASH and hepatocellular carcinoma. Similarly, whether increased Notch signaling precedes development of insulin resistance or heralds the transition to frank diabetes is unknown. In addition, it would be of interest to know whether interventions to reduce insulin resistance, or steatosis and associated inflammation (47,48), also reduce hepatic Notch signaling. Finally, whether higher Notch signaling seen in NASH patients reflects both hepatocyte and nonhepatocyte contribution requires clarification, because this may shed light on how Notch signals are transduced. These future studies will inform the question whether Notch may be a therapeutic target for insulin-resistance/diabetes or NASH.

It is premature, given these lingering questions and potential safety concerns, to propose a clinical trial with the use of Notch inhibitors, which are already in advanced clinical development for cancer (49,50), for treatment of T2D or NASH. This temptation exists, however, in the current era of pandemic obesity. Our data suggest the possibility of alternative uses for these existing therapeutics to combat the various faces of obesity-related metabolic disease in the 21st century.

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