Hepatic Notch Signaling Correlates with Insulin Resistance and Non-Alcoholic Fatty Liver Disease

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Running title: Notch Activation in Diabetes and Fatty Liver

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Abstract

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 both insulin resistance and hepatic steatosis. Here, we systematically survey Notch signaling in liver biopsy specimens, and show active Notch signaling in both 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, Notch activity showed independent positive association with both insulin resistance and hepatic steatosis. Notably, Notch activity showed stronger correlation with nonalcoholic fatty liver disease activity score and ALT 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.
Introduction

Obesity manifests as multiple pathologic states in 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 non-alcoholic fatty liver disease (NAFLD), in the presence of a predisposing genetic background (5). Steatosis may be associated with hepatocellular damage and necroinflammatory changes, defining non-alcoholic steatohepatitis (NASH), which predisposes to cirrhosis and hepatocellular cancer (1). Interestingly, NASH further exacerbates hepatic insulin resistance through activation of FoxO1, 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). There is no approved pharmacologic therapy for NALFD, and although there are multiple T2D therapies available, few show durability and long-term efficacy (7). As such, novel therapeutic directions are necessary to reduce overall obesity-related morbidity.

We have previously shown 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 which culminates in 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 liver, Notch proteins are constitutively expressed in multiple liver cell types (20,21), with increased expression in hepatocytes following 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 both hepatic glucose production (8) and triglyceride accumulation (9), lowering 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 inappropriatey stimulated by obesity, and may be manipulated to reduce obesity-related metabolic disease burden.
Based on 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, suggesting that it may represent a marker of the transition from simple steatosis 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 IRCCS Ca’ Granda, and 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. Needle liver biopsy was performed in all patients and formalin-preserved for histological and immunofluorescence analysis, and 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 Fondazione IRCCS Ca’ Granda Ospedale Policlinico di Milano, between 2006 and 2008. Indications for bariatric surgery included BMI>40 kg/m² or BMI>35 kg/m² in the presence of metabolic complications (type 2 diabetes, uncontrolled hypertension, severe dyslipidemia, obstructive sleep apnea). We excluded subjects with alcohol consumption >30/20 g/day for M/F (n=1), and chronic viral hepatitis (n=3). Fasting glucose, high-density lipoprotein (HDL) and total cholesterol, triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed the day of surgery, and needle liver biopsy (16G) 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 signalling and the regulation of lipid metabolism according to liver histology (27).

Hepatology clinic patients. We recruited 38 unrelated patients followed at the Metabolic Liver Diseases outpatient service, Fondazione Ca’ Granda IRCCS, who underwent percutaneous liver
biopsy because of suspected non-alcoholic fatty liver disease (NAFLD) due to persistently abnormal liver enzymes/serum ferritin 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 (>30/20 g/day for M/F), viral and autoimmune hepatitis, hereditary hemochromatosis, and alpha1-antitrypsin deficiency. Fasting glucose and insulin levels, HDL and total cholesterol, triglycerides, ALT and AST levels were assessed the day of biopsy. Patients were classified as insulin sensitive based on HOMA-IR < 2.5 [(fasting insulin (μU/mL) x fasting glucose (mmol/L))/22.5] (28) without history of impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), insulin resistant (HOMA-IR > 2.5 or either IFG or IGT but no diagnosis of diabetes) or type 2 diabetic (T2D).

**Histological analysis.** A single expert pathologist, unaware of gene expression and immunofluorescence data, evaluated all biopsies according to Kleiner et al (29), based on the determination of NAFLD activity score as result of the sum of steatosis severity (0-3), intralobular necro-inflammation (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 (NL), simple steatosis (SS), and non-alcoholic steatohepatitis (NASH).

**Immunofluorescence.** Paraffinized sections were de-paraffinized, rehydrated and stained as previously described (30). To determine Notch signal activation, tissues were stained with either Hey1 (#5714, 1:100 dilution) or HeyL (#10094, 1:150 dilution) antibodies from Millipore, and detected with donkey anti-rabbit alexafluor 488 (1:1000 dilution) or donkey anti-mouse alexafluor 594 (1:1000) from Invitrogen. Slides were mounted with Vectashield with DAPI (Vector laboratories). Images were captured with Nikon ECLIPSE E800 microscope, Nikon
DXM 1200 digital camera and Image ProPlus software. Staining was quantitated in a blinded fashion from 1 (low expression) to 4 (high expression), independently by three investigators, and scores averaged.

**Quantitative RT-PCR.** We isolated RNA with Trizol (Invitrogen), synthesized cDNA with Superscript III RT (Invitrogen), and performed qPCR 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 Rbp-Jk reporter luciferase construct previously described (8) then incubated in serum-free medium with variable glucose content in the presence or absence of 10nM insulin or 10nM 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 10nM insulin (Sigma) for 3 hours, with all experiments completed by 24 hours after isolation.

**Statistical analysis.** Results are shown as mean ± SEM. ANOVA was used for comparison of means between groups. Gene expression levels were correlated by Pearsons’. 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 when p was <0.05 (two-tailed).
Results

Human liver has a functional, evolutionarily conserved Notch signaling apparatus

To clarify the role of Notch signaling in post-development human liver, we characterized liver Notch and ligand expression in insulin-sensitive subjects with histologically normal liver. We found that in these subjects, all four Notch receptors were expressed, with relative higher levels of NOTCH1 and NOTCH2 (Figure 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 (Figure 1a, right panel), as well as mouse hepatocytes (8). Generally, expression of NOTCH1 and other Notch genes co-varied (Figure 1b) as well as with HES/HEY family target genes (Figure 1c), but showed no correlation with housekeeping (ACTB, GAPDH) and a non-significant trend towards negative correlation with Notch ligand expression (data not shown). This data shows that Notch signaling components are present in mature liver, and relative expression is evolutionarily conserved.

Hepatic Notch signaling correlates with G6PC/PCK1 expression and is increased in patients with Type 2 Diabetes

As Notch proteins and ligands are expressed in human liver, we hypothesized that Notch signaling may be 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 can be seen in Table 1, but the majority of subjects were female (76%), with SS or NASH (88%) and non-diabetic (79%). Expression of canonical Notch
target genes of the *HES/HEY* family positively correlated with hepatic expression of *G6PC* and *PCK1* ([Table 2](#) and [Figure 2a](#)). Of note, in severely obese patients we saw no correlation between Notch signaling and either age, gender or BMI (data not shown), which suggests that the correlations between *HES/HEY* and *G6PC/PCK1* was independent of overall adiposity, but correlations persisted or even strengthened when 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 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 ([Figure 2b](#)). HEY1 hepatocyte staining and *HEY1* gene expression were very well-correlated ([Figure 2c](#)), 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 ([Figure 2d](#) and 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 marked increase in HEY1 and HEYL staining in hyperglycemic patients ([Figure 2e, f](#) and data not shown). This data establishes that Notch signaling is present in adult hepatocytes, and increased in T2D.

**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 may precede development of frank diabetes in insulin resistance, we analyzed specimens from 38 consecutive outpatients undergoing percutaneous liver biopsy. Full demographics can be found in Table 1, but this group was overwhelmingly male (97%), with 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 (not shown), suggesting that Notch and gluconeogenic gene expression co-regulation 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 (HESI) and plasma insulin levels (not shown) and HOMA-IR, independent of confounding factors (Table 3), which was not mitigated by exclusion of diabetic patients (not shown). In multivariate logistic regression analysis, increased HESI expression was positively associated with the presence of insulin resistance of diabetes (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. This data establishes that Notch activation is found in the insulin-resistant liver, preceding frank hyperglycemia and 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 (Figure 3a). Similarly, endogenous Notch target expression was higher in primary mouse or human hepatocytes transiently exposed to hyperglycemia (Figure 3b, c). Insulin treatment had the opposite effect on Notch signaling, with insulin-treated cells showing decreased reporter activation (Figure 3d). Interestingly, the inhibitory effect of insulin, but not a synergistic effect of glucagon, was lost in cells chronically cultured in hyperglycemic conditions (Figure 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 (Figure 3f). Similarly, pharmacologic application of a novel Notch antagonist, an ectodomain “decoy” which quenches ligand-dependent Notch signaling (24), abrogated hyperglycemia-induced Notch-reporter activity (Figure 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 simple steatosis to NASH, and further still in insulin resistance (Figure 4a), or in patients with T2D (Figure 4b). Notch target staining was similarly increased in hepatocytes of NASH patients as compared to patients with simple steatosis (Figure 4c).
In fact, $HES1$ expression more closely correlated to measures of hepatic inflammation than steatosis, with stronger coefficients of correlation with both NAS and ALT levels than % steatosis (Figure 5a-c and Table 3). As such, patients with NAS score of 0-2, correlating with low risk of NASH (29,32), have lower Notch, ligand and $HES/HEY$ family gene expression than scores of 3 or higher (Figure 5d-f). Furthermore, 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 both insulin resistance as well as in NASH.
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 post-development role for Notch in regulation of obesity-induced insulin resistance/diabetes (8,26), as well as liver fat accumulation (9), but it was unclear whether this would translate to human disease. This work answers two important questions – hepatic Notch signaling is 1) present in human liver/hepatocytes, and 2) its activation tied to the metabolic state of the organism, with independent positive correlation with both measures of insulin resistance and hepatic steatosis/inflammation. In the insulin-resistant liver, Notch signaling appears to be inappropriately reactivated, reprising its developmental role and re-associating with its molecular partners from differentiation which drive 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 liver, increasing de novo lipogenesis and hepatic triglyceride (9). Mouse models with reduced hepatic glucose production often have compensatory fatty liver due to re-oriented 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 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 NAFLD activity score 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 prevalence approaching 30% in many populations (39), leaving a therapeutic dilemma as only a subset of patients progress to steatohepatitis (40). As such, increased Notch signaling may represent either a biomarker or even a causative factor for progression from simple steatosis 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 development of either 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. Additionally, 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 non-hepatocyte contribution requires clarification, as this may shed light on how Notch signals are transduced. These future studies will inform the question as to whether Notch may be a therapeutic target for either 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, 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 suggests 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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No potential conflicts of interest relevant to this article were reported.

U.B.P. designed and performed experiments, analyzed data, and wrote the manuscript. L.V. and C.J.S. analyzed data and wrote the manuscript. R.M. and C.K. designed and performed experiments and analyzed data. R.R. collected and managed biological samples for RNA analysis. M.M. selected and collected histological samples and reviewed histological samples. U.B.P. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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.

<table>
<thead>
<tr>
<th></th>
<th>Bariatric surgery</th>
<th>Hepatology clinic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NL</td>
<td>SS</td>
</tr>
<tr>
<td>n=</td>
<td>5 (12)</td>
<td>14 (33)</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>5/0</td>
<td>13/1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44±4</td>
<td>40±9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>40.8±10</td>
<td>40.5±10</td>
</tr>
<tr>
<td>Abd circ (cm)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>83±8</td>
<td>88±5</td>
</tr>
<tr>
<td>Insulin (IU/mL)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose tolerance IS (%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IS (%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IR (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T2D (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chol (mg/dL)</td>
<td>215±31</td>
<td>211±36</td>
</tr>
<tr>
<td>Trig (mg/dL)</td>
<td>99±54</td>
<td>155±95</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>73±11</td>
<td>52±9</td>
</tr>
<tr>
<td>Steatosis (%)</td>
<td>2±1</td>
<td>17±13</td>
</tr>
<tr>
<td>ALT (IU/ml)</td>
<td>18±5</td>
<td>26±9</td>
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</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>G6PC Rho</th>
<th>G6PC Pearson</th>
<th>PCK1 Rho</th>
<th>PCK1 Pearson</th>
</tr>
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<tbody>
<tr>
<td>HES1</td>
<td>+0.39</td>
<td>0.011</td>
<td>+0.47</td>
<td>0.0016</td>
</tr>
<tr>
<td>HES6</td>
<td>+0.24</td>
<td>NS</td>
<td>+0.33</td>
<td>0.018</td>
</tr>
<tr>
<td>HES7</td>
<td>+0.27</td>
<td>NS</td>
<td>+0.35</td>
<td>0.033</td>
</tr>
<tr>
<td>HEY1</td>
<td>+0.27</td>
<td>NS</td>
<td>+0.42</td>
<td>0.002</td>
</tr>
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</table>

NS: not significant
Table 3. *HES1* expression correlates independently with HOMA-IR as well as NAS scores.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>SE</th>
<th>Unadjusted P value*</th>
<th>Adjusted P value**</th>
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<tbody>
<tr>
<td>Age (per 10 years)</td>
<td>0.03</td>
<td>0.05</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.01</td>
<td>0.02</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (per 10 mg/dl increase)</td>
<td>0.00</td>
<td>0.02</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.02</td>
<td>0.01</td>
<td>0.002</td>
<td>0.03</td>
</tr>
<tr>
<td>ALT (per 10 UI/l increase)</td>
<td>0.07</td>
<td>0.02</td>
<td>0.003</td>
<td>-</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.11</td>
<td>0.03</td>
<td>0.002</td>
<td>-</td>
</tr>
<tr>
<td>%Steatosis (per 10% increase)</td>
<td>0.05</td>
<td>0.02</td>
<td>0.011</td>
<td>-</td>
</tr>
<tr>
<td>NAS</td>
<td>0.11</td>
<td>0.03</td>
<td>&lt;0.0001</td>
<td>0.03</td>
</tr>
</tbody>
</table>

SE: standard error; NAS: NAFLD activity score; NS: not significant; - : 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.
Figures

Figure 1. Notch pathway in human liver. (a) Notch receptor and ligand expression is similar in human (left) and mouse liver (right). (b) Hepatic NOTCH1 expression positively correlates with expression of other Notch receptors ($P<0.001$ by ANOVA for all comparisons) and (c) canonical Notch target HES1 ($P<0.001$ by ANOVA for all comparisons). Data show means ± SEM.

Figure 2. Notch activity in liver increases with hyperglycemia. (a) Correlation of Notch targets of the HES/HEY family with expression of genes controlling hepatic glucose production, Glucose-6-phosphatase (G6PC) and Phosphoenolpyruvate carboxykinase (PCK1). (b) Representative liver sections, stained with antibodies to either HEY1 (green, left) or HEYL (red, middle) and counter-stained with DAPI (blue), with merged image (right). Hepatic HEY1 expression correlates with (c) Hey1 staining ($P<0.001$ by ANOVA) and (d) HES1 expression ($P<0.001$ by ANOVA). (e) Immunofluorescence and (f) quantitation of staining for HEY1 in formalin-fixed liver sections from non-diabetic (non-T2D) and Type 2 Diabetic (T2D) patients undergoing liver biopsy during gastric bypass surgery. Very bright spots are auto-fluorescent erythrocytes, and are not included in quantitation. *$P<0.05$ vs. non-DM2 patients. Data show means ± SEM.

Figure 3. Notch activity in hepatocytes increases with hyperglycemia. (a) Notch-luciferase reporter (Csl-luc) expression in Hepa1c1c7 hepatoma cells, and Notch target gene expression in primary hepatocytes (b) from mice or (c) human donors is increased with transient exposure to normoglycemic (5mM glucose) or hyperglycemic (25mM glucose) conditions. (d) Insulin reduces Notch activation in Hepa1c1c7 hepatoma cells when cultured in normoglycemic, but not
(e) hyperglycemic conditions, 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, *P*<0.05, **P*<0.01, ***P*<0.001 vs. 5mM glucose or control (Cre- or GFP-transduced) cells. Data show means ± SEM.

**Figure 4.** Notch-dependent gene expression is progressively increased in insulin resistance and NAFLD severity. Quantitative PCR for *HES1* and other Notch target genes from human liver biopsy samples from patients with pathologically confirmed simple steatosis (SS), steatohepatitis (NASH) or normal liver fat, further subdivided as (a) insulin-sensitive (HOMA-IR < 2.5) vs. insulin-resistant (HOMA-IR > 2.5), ***P*<0.001 by ANOVA, or (b) Type 2 diabetic (T2D) patients. *P*<0.05 vs. T2D/SS. (c) Hepatocyte Notch target expression is increased in patients with NASH. Scale bars are 50 micrometers in length. Data show means ± SEM.

**Figure 5.** Notch activity correlates with hepatocyte necroinflammation. Liver *HES1* expression plotted against (b) NAFLD activity score (NAS), (c) serum ALT level and (d) %hepatic steatosis in patients undergoing percutaneous liver biopsy. (e) Notch target, (f) protein and (g) ligand gene expression is increased in liver from patients with higher NAS (NAS 3+) as compared to patients at low risk for steatohepatitis (NAS 0-2). *P*<0.05, **P*<0.01, ***P*<0.001 vs. NAS 0-2. Data show means ± SEM.
References


Figure 1

Human liver

Mouse liver

Notch receptor expression (fg/ng 18S)

Notch ligand expression (fg/ng 18S)
Figure 2

**a**

- **G6PC (fg/ng 18S)** vs. **HES1 (fg/ng 18S)**: $R^2 = 0.2934$
- **G6PC (fg/ng 18S)** vs. **HEY1 (fg/ng 18S)**: $R^2 = 0.2528$

- **PCK1 (fg/ng 18S)** vs. **HES1 (fg/ng 18S)**: $R^2 = 0.5726$
- **PCK1 (fg/ng 18S)** vs. **HEY1 (fg/ng 18S)**: $R^2 = 0.5681$
**Figure 2 (cont’d)**

- **c**
  - R² = 0.5724
  - Graph showing HEY1 expression (fg/ng 18S) vs. HEY1 staining (AU)

- **d**
  - R² = 0.4598
  - Graph showing HES1 expression (fg/ng 18S) vs. HEY1 expression (fg/ng 18S)

- **e**
  - Images of HEY1 staining in non-diabetic and Type 2 Diabetic samples

- **f**
  - Bar graph comparing Hey1 staining (AU) between non-T2D and T2D groups
  - Non-T2D: [staining value]
  - T2D: [staining value]
  - * indicates a significant difference

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Figure 4

(a) Graph showing the expression of HES1 in different states: Normal, SS, NASH, SS, and NASH. The x-axis represents different states, and the y-axis represents HES1 expression in fg/ng 18S. The graph indicates a significant difference among states, as denoted by "***".

(b) Bar graph comparing the Notch target expression in AU between different conditions: T2D/SS, T2D/NASH. The bars for "HES1", "HEY1", "HEY2", and "HEYL" are shown, with "*" indicating statistical significance.

(c) Images showing Hey1 and HeyL expression in normal, steatosis, NASH, and NASH/T2D conditions. The images display the expression levels and locations of the proteins in various tissue samples.
Figure 5

(a) HES1 (fg/ng 18S) vs. NAFLD activity score, \( R^2 = 0.3164 \)

(b) HES1 (fg/ng 18S) vs. ALT (U/ml), \( R^2 = 0.2104 \)

(c) HES1 (fg/ng 18S) vs. Steatosis (%), \( R^2 = 0.1573 \)

(d) Notch target expression (AU) for NAS 0-2 and NAS 3+

(e) Notch receptor expression (fg/ng 18S) for NAS 0-2 and NAS 3+

(f) Notch ligand expression (fg/ng 18S) for NAS 0-2 and NAS 3+