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### Authors

Sundaram, Shikha

Swiderska-Syn, Marzena

Sokol, Ronald

et al.

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# Nocturnal Hypoxia Activation of the Hedgehog Signaling Pathway Affects Pediatric Nonalcoholic Fatty Liver Disease Severity

Shikha S. Sundaram,<sup>1</sup> Marzena Swiderska-Syn,<sup>2</sup> Ronald J. Sokol,<sup>1</sup> Ann C. Halbower,<sup>3</sup> Kelley E. Capocelli,<sup>4</sup> Zhaoxing Pan,<sup>5</sup> Kristen Robbins,<sup>1</sup> Brian Graham,<sup>6</sup> and Anna Mae Diehl<sup>2</sup>

Chronic intermittent hypoxia and hedgehog (Hh) pathway dysregulation are associated with nonalcoholic fatty liver disease (NAFLD) progression. In this study, we determined the relationship between obstructive sleep apnea (OSA)/nocturnal hypoxia and Hh signaling in pediatric NAFLD. Adolescents with histologic NAFLD (n = 31) underwent polysomnogram testing, laboratory testing, and Sonic Hh (SHh), Indian hedgehog (IHh), glioblastoma-associated oncogene 2 (Gli2), keratin 7 (K7),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) immunohistochemistry. Aspartate aminotransferase (AST) correlated with SHh,  $r = 0.64$ ; Gli2,  $r = 0.4$ ;  $\alpha$ -SMA,  $r = 0.55$ ; and K7,  $r = 0.45$  ( $P < 0.01$ ), as did alanine aminotransferase (ALT) (SHh,  $r = 0.51$ ; Gli2,  $r = 0.43$ ;  $\alpha$ -SMA,  $r = 0.51$ ;  $P < 0.02$ ). SHh correlated with NAFLD activity score ( $r = 0.39$ ), whereas IHh correlated with inflammation ( $r = -0.478$ ) and histologic grade ( $r = -0.43$ );  $P < 0.03$ . Subjects with OSA/hypoxia had higher SHh ( $4.0 \pm 2.9$  versus  $2.0 \pm 1.5$ ), Gli2 ( $74.2 \pm 28.0$  versus  $55.8 \pm 11.8$ ), and  $\alpha$ -SMA ( $6.2 \pm 3.3$  versus  $4.3 \pm 1.2$ ); compared to those without ( $P < 0.03$ ). OSA severity correlated with SHh ( $r = 0.31$ ;  $P = 0.09$ ) and Gli2 ( $r = 0.37$ ;  $P = 0.04$ ) as did hypoxia severity, which was associated with increasing SHh ( $r = -0.53$ ), Gli2 ( $r = -0.52$ ),  $\alpha$ -SMA ( $r = -0.61$ ), and K7 ( $r = -0.42$ );  $P < 0.02$ . Prolonged O<sub>2</sub> desaturations  $< 90\%$  also correlated with SHh ( $r = 0.55$ ) and Gli2 ( $r = 0.61$ );  $P < 0.05$ . **Conclusion:** The Hh pathway is activated in pediatric patients with NAFLD with nocturnal hypoxia and relates to disease severity. Tissue hypoxia may allow for functional activation of HIF-1 $\alpha$ , with induction of genes important in epithelial-mesenchymal transition, including SHh, and NAFLD progression. (*Hepatology Communications* 2019;3:883–893).

**N**onalcoholic fatty liver disease (NAFLD), characterized by abnormal lipid deposition in hepatocytes in the absence of excess alcohol intake, is the most common chronic liver disease affecting both children and adults and parallels the obesity epidemic.<sup>(1)</sup> Alarming, 9.6% of all children and 38% of children with obesity are affected by NAFLD across a spectrum of disease, including isolated hepatic

*Abbreviations:*  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; AHI, apnea-hypopnea index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CIH, chronic intermittent hypoxia; Gli2, glioblastoma-associated oncogene 2; Hh, hedgehog; HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; HPF, high-power field; IHh, Indian hedgehog; K7, keratin 7; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NS, not significant; OSA, obstructive sleep apnea; Ptc, Patched; SaO<sub>2</sub>, O<sub>2</sub> saturation; SHh, sonic hedgehog; Smo, Smoothened.

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steatosis, nonalcoholic steatohepatitis (NASH) (defined as steatosis, hepatocyte ballooning, and inflammation), and cirrhosis.<sup>(1,2)</sup> NASH progresses to liver fibrosis and cirrhosis in approximately 20% of cases and is associated with hepatocellular carcinoma in adults.<sup>(1,3)</sup>

Mounting evidence implicates obesity-related obstructive sleep apnea (OSA) and chronic intermittent hypoxia (CIH) in NAFLD progression. Patients with OSA/CIH are exposed to repeated cycles of hypoxia alternating with normoxia, similar to the pathophysiology of ischemia/reperfusion injury.<sup>(4,5)</sup> Obese mice fed a high-fat high-cholesterol diet develop hepatic steatosis. Subsequent exposure to CIH leads to significant increases in alanine aminotransferase (ALT) and histologic evidence of hepatic inflammation and fibrosis.<sup>(6,7)</sup> Adults who are morbidly obese and have moderate to severe OSA and CIH demonstrate more significant hepatic inflammation than those without hypoxia.<sup>(8)</sup> Pediatric patients with NAFLD with OSA/hypoxia also have more advanced liver disease and fibrosis than those without OSA/hypoxia.<sup>(9,10)</sup> Moreover, we have recently reported that OSA/nocturnal hypoxia-induced oxidative stress, propagated through reactive oxygen species generation, promotes pediatric NAFLD progression.<sup>(10)</sup> Although these data support a role for OSA/hypoxia in NASH pathogenesis, the mechanism underlying this relationship has not been elucidated.

There is increasing evidence supporting a role for dysregulation of the hedgehog (Hh) pathway in the pathogenesis of both adult and pediatric NAFLD.<sup>(11,12)</sup> Hh, a morphogenic signaling pathway, controls organogenesis during organ development and is usually quiescent in adolescents and adults. Hh ligand

activates Hh signaling by engaging Patched (Ptc), a transmembrane surface receptor on cells responsive to Hh.<sup>(12-14)</sup> Hh ligands bind to Ptc, thereby preventing Ptc from inhibiting Smoothened (Smo). Activated Smo subsequently controls the cellular accumulation and nuclear localization of glioblastoma transcription factors, which in turn regulate the expression of other Hh-regulated genes critical for the proliferation, differentiation, and survival of Hh-responsive cells.<sup>(13,14)</sup> Although typically silent, Hh may be reactivated during injury that stimulates Hh ligand production. In the liver injury incited in both adult and pediatric NAFLD, activation of the Hh pathway is a dominant mechanism by which fibro-inflammatory repair occurs.<sup>(11,12)</sup>

In adult mice, hepatic hypoxia induces expression of the Hh ligand sonic hedgehog (SHh) and the pathway activity marker Ptc1, thereby inciting a systemic Hh response.<sup>(15)</sup> The impact of hypoxia on Hh pathway activation in human liver disease, however, is unknown. This study was conducted to understand the relationship between OSA/nocturnal hypoxia and dysregulation of the Hh signaling pathway in pediatric NAFLD. We hypothesized that OSA and CIH would be associated with up-regulation of the Hh pathway, with evidence of increasing histologic severity of pediatric NAFLD.

## Patients and Methods

Eligible study subjects were children cared for at Children's Hospital Colorado Pediatric Liver Center between June 2009 and January 2014. The subjects had

### ARTICLE INFORMATION:

From the <sup>1</sup>Section of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics and the Digestive Health Institute, Children's Hospital Colorado and University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, CO; <sup>2</sup>Duke University, Durham, NC; <sup>3</sup>Pulmonary Section, Department of Pediatrics, Children's Hospital Colorado and University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, CO; <sup>4</sup>Pediatric Pathology, University of Colorado School of Medicine, Aurora, CO; <sup>5</sup>Department of Biostatistics and Informatics, Colorado School of Public Health, Aurora, CO; <sup>6</sup>Program in Translational Lung Research, Department of Medicine, University of Colorado School of Medicine, Aurora, CO.

### ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Shikha S. Sundaram, M.D., M.S.C.I.  
Digestive Health Institute, Children's Hospital Colorado  
13123 E. 16th Avenue, B290

Aurora, CO 80045  
E-mail: shikha.sundaram@childrenscolorado.org  
Tel.: +1-720-777-6669

suspected NAFLD and were scheduled to undergo a clinically indicated liver biopsy. Inclusion criteria were male and female children, ages 8 through 18 years, and Tanner stages 2-4. Exclusion criteria for the study were the presence of Wilson's disease, alpha-1-antitrypsin deficiency, viral hepatitis, autoimmune hepatitis, other known chronic liver disease, cholelithiasis or use of corticosteroids, anticonvulsants, sedatives, drugs that promote or reduce insulin resistance (including metformin, insulin sensitizers, and thiazolidinediones), or other treatments known to induce hepatic steatosis (amiodarone or parenteral nutrition) in the 2 weeks prior to the liver biopsy. Additional exclusion criteria included regular tobacco or alcohol use, insulin-dependent diabetes, neuromuscular disorders, genetic or craniofacial abnormalities, and current use of continuous positive airway pressure. Data from a subset of these patients have been reported.<sup>(9,10)</sup> This study was approved by the Colorado Multiple Institutional Review Board, and informed written consent was obtained from parents/guardians and written assent from all subjects.

Demographic and medical history data were obtained. Heights, weights, and body mass index (BMI) *z* scores (based on age and sex data from the Centers for Disease Control and Prevention) were determined.<sup>(16)</sup> Subjects with suspected NAFLD underwent liver biopsy for clinical indications by standard percutaneous technique. In our center, we suspect NAFLD in children who are overweight or obese (BMI >85% for age and sex), with chronically elevated aminotransferases, with negative screening for other chronic liver diseases. Liver histology (hematoxylin and eosin and Masson's trichrome stains) was reviewed and scored by a single pediatric pathologist blinded to subject information. Biopsies with histologically confirmed NAFLD (defined as  $\geq 5\%$  of hepatocytes containing macrovesicular fat) were assigned a grade of necro-inflammation (0-3) and a stage of fibrosis (0-4) based on the standard histologic criteria described by Brunt et al.<sup>(17)</sup> Each biopsy was also scored for NASH Clinical Research Network criteria<sup>(18)</sup>: steatosis grade 0 (<5% of hepatocytes containing macrovesicular fat), grade 1 (5%-33%), grade 2 (34%-66%), and grade 3 (>66%); lobular inflammation grade 0 (no foci of inflammation), grade 1 (<2 foci per high-power field [HPF]), grade 2 (2-4 foci per HPF), and grade 3 (>4 foci per HPF); and

ballooning degeneration grade 0 (none), grade 1 (few balloon cells), and grade 2 (many/prominent balloon cells). The NAFLD activity score (NAS) was calculated by summing the individual scores for steatosis, lobular inflammation, and ballooning degeneration.<sup>(18)</sup> Subjects were also classified as having definite NASH (NAS  $\geq 5$ ), not NASH, or borderline NASH (NAS  $\leq 4$ ).<sup>(19)</sup> Hepatic fibrosis was scored as stage 0 (none), stage 1 (mild to moderate perisinusoidal or portal/periportal fibrosis only), stage 2 (zone 3 and periportal fibrosis), stage 3 (bridging fibrosis), or stage 4 (cirrhosis).<sup>(18,20)</sup> Furthermore, subjects were defined as either type 1 (classic adult pattern), type 2 (portal-based histology), or an overlap of the two NASH histologic subtypes.<sup>(20)</sup> Immunostaining for hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) was carried out using the Dako Cytomation CSA system (Dako Corporation, Santa Clara, CA) for mouse primary antibodies (K1500). Antigen retrieval was performed using citrate buffer for 30 minutes. The primary antibody was Novus Biological NB 100-123, used at a 1:8,000 dilution, and slides were counterstained with hematoxylin. HIF-1 $\alpha$  staining intensity was graded from 0 (no staining) to 3 (significant staining), and the location of staining was noted to be nuclear, cytoplasmic, or both.

Liver biopsy sections were stained to detect SHh, Indian hedgehog (IHh), glioblastoma-associated oncogene 2 (Gli2), and markers of progenitors (keratin 7 [K7]) and myofibroblasts ( $\alpha$ -smooth muscle actin [ $\alpha$ -SMA]), using the Dako Envision System according to the manufacturer's protocol. Immunohistochemistry was performed as described.<sup>(21)</sup> Briefly, formalin-fixed paraffin-embedded liver tissue was cut into 5- $\mu$ m sections and placed on glass slides. Sections were deparaffinized with xylene, dehydrated with ethanol, and incubated with 3% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval was performed by heating in 10 mM sodium citrate buffer (pH 6.0). Sections were blocked in Dako protein block (X9090; Dako) and followed by incubation with primary antibodies (SHh, Abcam-100639; IHh, Abcam-39634; Gli2, GenWay Biotech EB-3B44;  $\alpha$ -SMA, Abcam-32575; and K7, Dako-M7018). Horseradish peroxidase (HRP)-conjugated anti-rabbit (K4003; Dako) and HRP-conjugated anti-mouse (K4001; Dako) secondary antibodies were used to visualize target proteins. 3,3'-diaminobenzidine reagent (K3466; Dako) was applied in the detection procedure. Tissue

sections were counterstained with Aqua Hematoxylin-INNOVEX (Innovex Biosciences). Negative controls included liver specimens exposed to 1% bovine serum albumin instead of the respective primary antibodies. The number of detectable SHh, IHh, Gli2,  $\alpha$ -SMA, and K7 immunoreactive cells was quantified by counting 20 randomly chosen 20 $\times$  fields per section per slide. Additionally, the histologic locations of SHh and Gli2 staining were determined.

Following pathology review of initial liver biopsies, subjects with histologically confirmed NAFLD underwent a standard multichannel sleep study (polysomnogram), which was scored by a trained technician and interpreted by a single sleep medicine physician, both of whom were blinded to liver biopsy results. The following data were analyzed: total sleep time, percentage of rapid eye movement (REM) sleep, apnea-hypopnea index (AHI), oxygen nadir, percentage of time O<sub>2</sub> saturation (SaO<sub>2</sub>)  $\leq$ 90%, and oxygen desaturation index (the number of SaO<sub>2</sub> drops below 95% by pulse oximeter). OSA was defined as an AHI  $>$ 2.0, indicating total apneas and hypopneas per hour of total sleep time.<sup>(22,23)</sup> Apnea was defined as cessation of airflow over  $\geq$ 2 attempted respiratory cycles. Hypopnea was defined as a decrease in nasal pressure of  $\geq$ 50%, with a corresponding decrease in SaO<sub>2</sub> of  $\geq$ 3% and/or arousal. Hypoxia was defined as SaO<sub>2</sub>  $<$ 90% for  $\geq$ 1% of total sleep time.<sup>(24)</sup>

The morning after the polysomnogram, fasting blood specimens were collected for serum ALT, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase, ultrasensitive C-reactive protein, total cholesterol, triglyceride, high-density lipoprotein, glucose, and insulin. Fasting glucose and insulin levels were used to calculate insulin resistance at baseline using the homeostasis model assessment of insulin resistance.<sup>(25)</sup>

Statistical analyses were performed using SAS 9.3 software (SAS Institute, Inc., Cary, NC). Descriptive statistics are presented as either mean  $\pm$  SD for continuous measures or percentages for categorical responses. Two-sample *t* test, chi-squared test, or Fisher's exact test were used, as appropriate, to assess differences between subjects with and without OSA/hypoxia. Pearson or Spearman correlation coefficients, as appropriate, were used to quantify the relationships between ALT, AST, and histologic parameters with polysomnographic parameters and Hh makers.  $P < 0.05$  was considered statistically significant.

## Results

### RELATIONSHIP OF OSA/HYPOXIA TO NAFLD

A total of 31 subjects with liver biopsy-confirmed NAFLD were studied. These adolescents (mean age, 13.0  $\pm$  1.9 years) were 65% male subjects, 87% Hispanic, and had obesity (mean BMI *z* score, 2.2  $\pm$  0.3). Twenty-one subjects (68%) met our study criteria for OSA and/or hypoxia: 1 had isolated hypoxia, 12 had isolated OSA, and 8 had both OSA and hypoxia. Subjects with and without OSA/hypoxia had similarly elevated aminotransferases, inflammatory markers, triglycerides, and evidence of insulin resistance (Table 1). Although total serum cholesterol concentration was higher in those with OSA/hypoxia ( $P = 0.03$ ), this did not impact NAFLD disease severity. In all subjects with NAFLD, polysomnograms were of adequate length with  $>$ 12% total sleep time in rapid eye movement (REM) sleep, allowing all studies to be considered valid. Mean total sleep time and percentage of REM sleep were similar between groups. Subjects with NAFLD with OSA/hypoxia had a significantly higher ( $P < 0.001$ ) mean AHI score (9.0  $\pm$  8.2) versus those without OSA/hypoxia (0.9  $\pm$  0.6), suggesting moderate to severe sleep-disordered breathing in affected subjects (Table 1). Those with OSA/hypoxia also had significantly lower oxygen nadirs versus those without OSA/hypoxia (82.6%  $\pm$  6.3% versus 87.8%  $\pm$  2.0%;  $P = 0.004$ ) and spent a greater percentage of sleep time with SaO<sub>2</sub>  $<$ 90% (2.2  $\pm$  3.8 versus 0.1  $\pm$  0.2;  $P = 0.02$ ) (Table 1).

### LIVER INJURY AND OSA/HYPOXIA

Both AST and ALT values were similar in subjects with NAFLD with and without OSA/CIH ( $P$  was not significant [NS]). Liver histology scores for steatosis, inflammation, ballooning degeneration, NAS summary score (mean NAS, 4.81  $\pm$  1.29 versus 5.01  $\pm$  1.10), and histologic grade were also similar between those with and without OSA/hypoxia. However, subjects with NAFLD with OSA/hypoxia trended toward more severe fibrosis (62% stages 0-2; 38% stage 3) than those without OSA/hypoxia (100% stages 0-2;  $P = 0.08$ ). Increasing time with SaO<sub>2</sub>  $<$ 90% was also associated with higher hepatic steatosis ( $r = 0.4$ ;  $P = 0.0008$ ), histologic grade of inflammation ( $r = 0.36$ ;  $P = 0.04$ ), and NAS ( $r = 0.35$ ;  $P = 0.055$ ).

**TABLE 1. DEMOGRAPHIC AND CLINICAL PARAMETERS OF SUBJECTS WITH NAFLD WITH AND WITHOUT OSA/HYPOXIA**

Clinical Parameter	NAFLD With OSA/Hypoxia (n = 21)	NAFLD Without OSA/Hypoxia (n = 10)	P Value	Normal Values
Mean age	13.0 ± 1.9	13.1 ± 2.3	0.8	–
Male sex (%)	67	50	0.4	–
Hispanic ethnicity (%)	86	100	0.2	–
BMI z score	2.3 ± 0.3	2.1 ± 0.3	0.3	–
ALT (IU/L)	131 ± 109	93 ± 63	0.3	10-45 IU/L
AST (IU/L)	79 ± 55	58 ± 27	0.2	15-40 IU/L
Cholesterol (mg/dL)	157 ± 38	121 ± 43	0.03	≤170 mg/dL
Triglycerides (mg/dL)	148 ± 66	139 ± 59	0.7	≤150 mg/dL
HOMA-IR	10.0 ± 6.7	8.1 ± 9.0	0.5	<2.60
Adiponectin (µg/mL)	6.6 ± 3.4	6.6 ± 2.9	1.0	3.5-14 µg/mL
Leptin (ng/mL)	30.6 ± 15.5	29.9 ± 11.1	0.5	2.0-5.6 ng/mL
CRP (mg/dL)	3.0 ± 3.5	1.8 ± 1.2	0.2	<0.5 mg/dL
Uric acid (mg/dL)	6.6 ± 1.5	5.9 ± 1.3	0.2	2.3-5.4 mg/dL
Sleep parameters				
Apnea-hypopnea index	9.0 ± 8.2	0.9 ± 0.6	0.0002	–
Oxygen nadir	82.6 ± 6.4	87.8 ± 2.9	0.004	–
% Time SaO <sub>2</sub> <90%	2.2 ± 3.8	0.1 ± 0.2	0.02	–

Abbreviations: CRP, C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance.

There were no differences in the distribution of histologic type 1 versus type 2 NAFLD in subjects with and without OSA/hypoxia. Subjects with NAFLD with definite NASH (NAS histologic score, ≥5; n = 20) had more severe sleep-disordered breathing compared to those without definite NASH (NAS score, <5; n = 11). Subjects with definite NASH trended toward higher ( $P = 0.07$ ) mean AHI scores ( $7.9 \pm 9.2$ ) than those without definite NASH ( $3.7 \pm 2.8$ ). Subjects with definite NASH spent significantly more time ( $P = 0.03$ ) with SaO<sub>2</sub> <90% ( $2.3 \pm 3.9$ ) than those without definite NASH ( $0.19 \pm 0.35$ ). There were no differences in oxygen nadir or oxygen desaturation index (data not shown). There were no differences noted in HIF-1 $\alpha$  immunohistochemistry staining intensity in subjects with NAFLD with (mean,  $1.92 \pm 0.86$ ) and without OSA/hypoxia ( $1.78 \pm 0.83$ ) or location. There were also no associations found between HIF-1 $\alpha$  immunohistochemistry staining intensity or location and severity of OSA/hypoxia, as indicated by AHI, oxygen nadir, or time with SaO<sub>2</sub> <90%.

## MUTUAL ASSOCIATION OF Hh SIGNALING MARKERS

The presence of SHh on immunohistochemistry was strongly correlated with the presence of Gli2

( $r = 0.56$ ;  $P = 0.001$ ), K7 ( $r = 0.71$ ;  $P = <0.001$ ), and  $\alpha$ -SMA ( $r = 0.70$ ;  $P < 0.001$ ) but not IHh. The presence of IHh on immunohistochemistry also did not correlate with Gli2, K7, or  $\alpha$ -SMA. In addition, Gli2 correlated strongly with K7 ( $r = 0.67$ ;  $P < 0.001$ ) and  $\alpha$ -SMA ( $r = 0.58$ ;  $P = 0.001$ ) as did K7 and  $\alpha$ -SMA ( $r = 0.74$ ;  $P < 0.001$ ).

## LIVER INJURY AND Hh SIGNALING

Liver injury was closely associated with markers of Hh activation. AST correlated strongly with SHh ( $r = 0.64$ ;  $P = 0.0001$ ), Gli2 ( $r = 0.47$ ;  $P = 0.007$ ),  $\alpha$ -SMA ( $r = 0.55$ ;  $P = 0.002$ ), and K7 ( $r = 0.45$ ;  $P = 0.01$ ). Similarly, ALT correlated strongly with SHh ( $r = 0.51$ ;  $P = 0.003$ ), Gli2 ( $r = 0.43$ ;  $P = 0.02$ ), and  $\alpha$ -SMA ( $r = 0.51$ ;  $P = 0.005$ ) but not K7 ( $r = 0.30$ ;  $P = 0.09$ ). SHh correlated with the NAS summary score ( $r = 0.39$ ;  $P = 0.03$ ) but not with steatosis, lobular inflammation, hepatocyte ballooning, or fibrosis. IHh was inversely correlated with inflammation ( $r = -0.478$ ;  $P = 0.01$ ) and histologic grade ( $r = -0.43$ ;  $P = 0.02$ ) but not with steatosis, hepatocyte ballooning, NAS summary score, or fibrosis. Neither Gli2 nor K7 correlated with steatosis, inflammation, hepatocyte ballooning, NAS summary score, or fibrosis ( $P$  was NS).

Finally,  $\alpha$ -SMA correlated strongly with both the NAS summary score ( $r = 0.43$ ;  $P = 0.02$ ) and fibrosis stage ( $r = 0.41$ ;  $P = 0.03$ ) but not steatosis, inflammation, and hepatocyte ballooning. In addition, subjects with NAFLD inflammation grade<sup>(17)</sup> 3-5 had higher  $\alpha$ -SMA (mean,  $7.14 \pm 3.32$ ) and SHh ( $4.48 \pm 2.75$ ) than subjects with NAFLD inflammation grade 0-2 ( $\alpha$ -SMA,  $4.90 \pm 2.39$ ;  $P = 0.05$ ; and SHh,  $2.47 \pm 2.44$ ;  $P = 0.04$ ). Subjects with type 1 NASH had slightly higher SHh (mean,  $3.74 \pm 2.35$ ) compared to type 2 histologic NASH ( $1.88 \pm 1.86$ ;  $P = 0.058$ ). There were no differences in IHh, Gli2, K7, or  $\alpha$ -SMA between subjects with histologic type 1 versus type 2 NAFLD ( $P$  was NS).

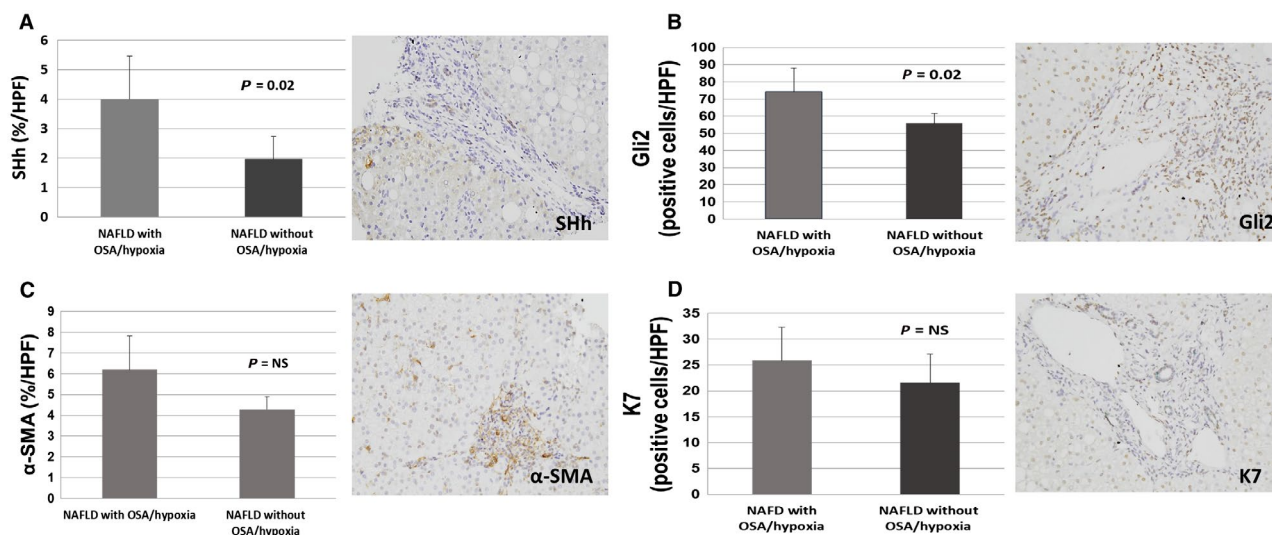
## OSA/HYPOXIA AND Hh SIGNALING

Subjects with OSA/hypoxia had higher mean SHh ( $4.0 \pm 2.9$  versus  $2.0 \pm 1.5$ ), Gli2 ( $74.2 \pm 28.0$  versus  $55.8 \pm 11.8$ ), and  $\alpha$ -SMA immunohistochemistry scores ( $6.2 \pm 3.3$  versus  $4.3 \pm 1.2$ ) but not IHh ( $1.1 \pm 0.9$  versus  $0.9 \pm 0.8$ ) or K7 ( $25.8 \pm 12.8$  versus  $21.5 \pm 11.1$ ) (Fig. 1). The severity of OSA, as measured by AHI, was positively correlated with both SHh ( $r = 0.31$ ;  $P = 0.09$ ) and Gli2 ( $r = 0.37$ ;  $P = 0.04$ ) (Fig. 2) but not IHh, K7, or  $\alpha$ -SMA. The severity of hypoxia, as indicated by worsening

oxygen nadir, was also significantly associated with increasing SHh ( $r = -0.53$ ;  $P = 0.002$ ), Gli2 ( $r = -0.52$ ;  $P = 0.003$ ),  $\alpha$ -SMA ( $r = -0.61$ ;  $P = 0.006$ ), and K7 ( $r = -0.42$ ;  $P = 0.02$ ) (Fig. 3). Prolonged duration of hypoxia was also associated with prominent Hh signaling, as demonstrated by significant correlations between increasing percentage of sleep time with  $\text{SaO}_2 < 90\%$  and SHh ( $r = 0.5$ ;  $P = 0.002$ ), Gli2 ( $r = 0.61$ ;  $P = 0.0003$ ),  $\alpha$ -SMA ( $r = 0.7$ ;  $P < 0.0001$ ), and K7 ( $r = 0.36$ ;  $P = 0.048$ ) (Fig. 4). There were no associations between HIF-1 $\alpha$  immunohistochemistry and SHh, Gli2, or K7.

## OSA/HYPOXIA AND HISTOLOGIC LOCATION OF SHh AND Gli2

A majority (88%) of subjects with NAFLD and OSA/hypoxia demonstrated an intense ductular reaction compared to only 40% of those without OSA/hypoxia ( $P = 0.007$ ). SHh positivity was visualized in three relatively discrete histologic patterns: (1) SHh+ bile duct/ductular cells, (2) SHh+ portal/periportal hepatocytes, and (3) a combination of SHh+ bile duct/ductular cells and portal/periportal hepatocytes. Subjects with OSA/hypoxia demonstrated a predominant pattern of SHh positivity in portal/periportal hepatocytes (78%), with 11% in bile duct/ductular cells and 11% in both areas. In contrast,



**FIG. 1.** Comparison of subjects with NAFLD and OSA/hypoxia. Subjects with NAFLD with OSA/hypoxia have higher (A) SHh, (B) Gli2, and (C)  $\alpha$ -SMA but not (D) K7. Representative immunohistochemistry (magnification  $\times 20$ ) demonstrates (A) SHh, (B) Gli2, (C)  $\alpha$ -SMA, and (D) K7 staining in subjects with NAFLD with OSA/hypoxia. Data represent mean  $\pm$  SD.

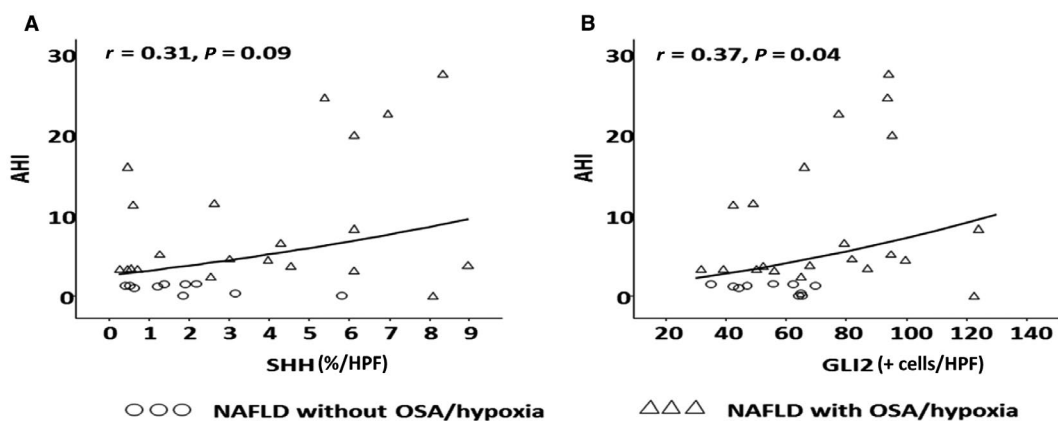


FIG. 2. Association of obstructive sleep apnea and hedgehog signaling. The severity of obstructive sleep apnea as measured by the AHI is associated with increased (A) SHh and (B) Gli2.

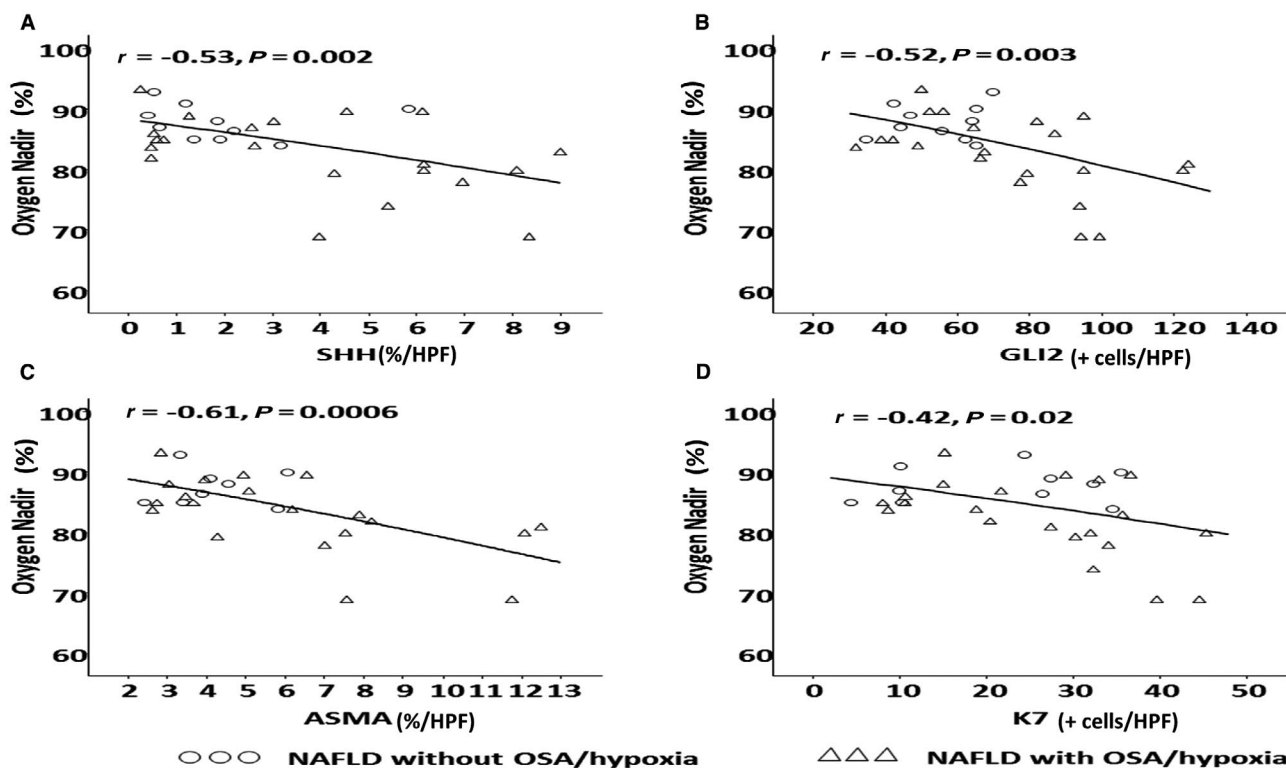
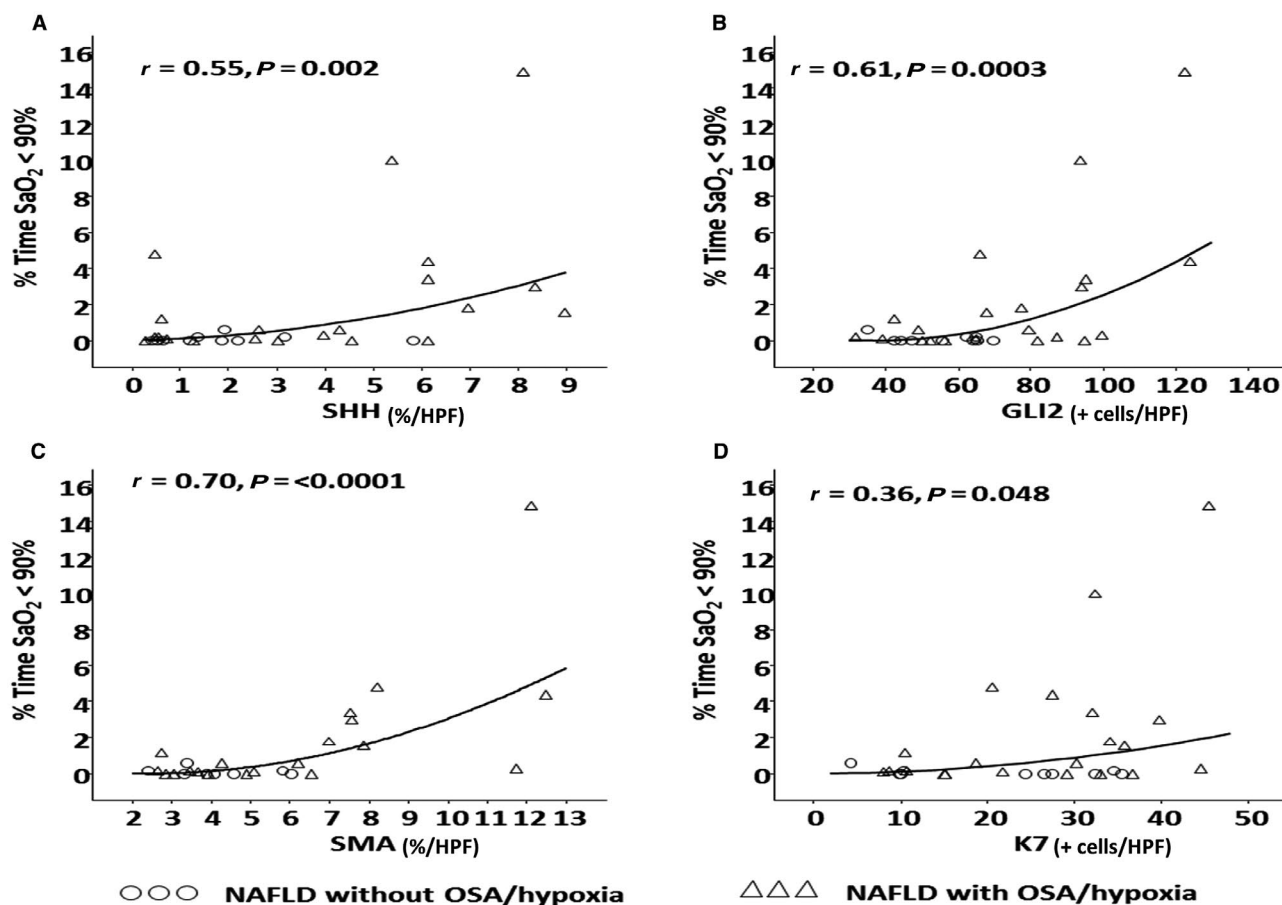


FIG. 3. Association of hypoxia and hedgehog signaling. The severity of hypoxia as measured by worsening oxygen nadir is associated with increased Hh signaling, including (A) SHh, (B) GLi2, (C)  $\alpha$ -SMA, and (D) K7.

SHh positivity in subjects without OSA/hypoxia was predominantly in bile duct/ductular cells (60%), with only 40% in the portal/periportal hepatocytes ( $P = 0.01$ ). Similarly, Gli2-positive cells were located

in the portal/periportal areas in 84% of those with OSA/hypoxia, with the remaining 16% showing Gli2 positivity diffusely across the portal/periportal, bile duct/ductular, and lobular locations. In comparison,





**FIG. 4.** Association of duration of hypoxia and hedgehog signaling. An increasing duration of hypoxia is associated with prominent Hh signaling in pediatric NAFLD, including (A) SHh, (B) Gli2, (C)  $\alpha$ -SMA, and (D) K7.

Gli2 positivity was located in the portal/periportal areas in 50% of subjects without OSA/hypoxia and diffusely across portal/periportal, bile duct/ductular, and lobular locations in 50% of subjects ( $P = 0.05$ ).

## Discussion

In this study, we demonstrated that the Hh pathway is activated in pediatric NAFLD and relates not only to NAFLD disease severity but importantly to the severity of both OSA and severity and duration of chronic intermittent nocturnal hypoxia. The results of this study further implicate OSA and CIH as important factors that may promote the progression of pediatric NASH through, at least in part, the Hh pathway.

Increasing evidence implicates abnormal regulation of the Hh pathway in the pathogenesis and

progression of NAFLD. Hh, a morphogenic signaling pathway quiescent in the adult liver, can be reactivated by injury that stimulates the growth of cells involved in hepatic wound healing, including immune, stellate, and progenitor cells.<sup>(13,26,27)</sup> In adults with NASH, ballooned hepatocytes produce Hh ligands; the number of ligands and Hh-responsive (Gli2-positive) cells correlate with both the severity of hepatic inflammation and fibrosis.<sup>(28)</sup> Similarly, children with NAFLD exhibit increasing SHh expression associated with the degree of steatosis and fibrosis.<sup>(27)</sup> Inflammation and fibrosis are also related to the number of Gli2-positive cells, and inflammation was associated with the number of K7 cells (a progenitor cell marker<sup>(27)</sup>). We expand on this work, showing that liver injury, as measured by aminotransferase elevation, was closely associated with markers of Hh activation. Moreover, hepatic SHh immunohistochemistry correlated

strongly with the NAS summary score, although not with individual measures of steatosis, inflammation, or ballooning. These data collectively suggest that activation of the Hh pathway during liver injury in pediatric NAFLD may be a crucial mechanism driving the fibro-inflammatory repair response.

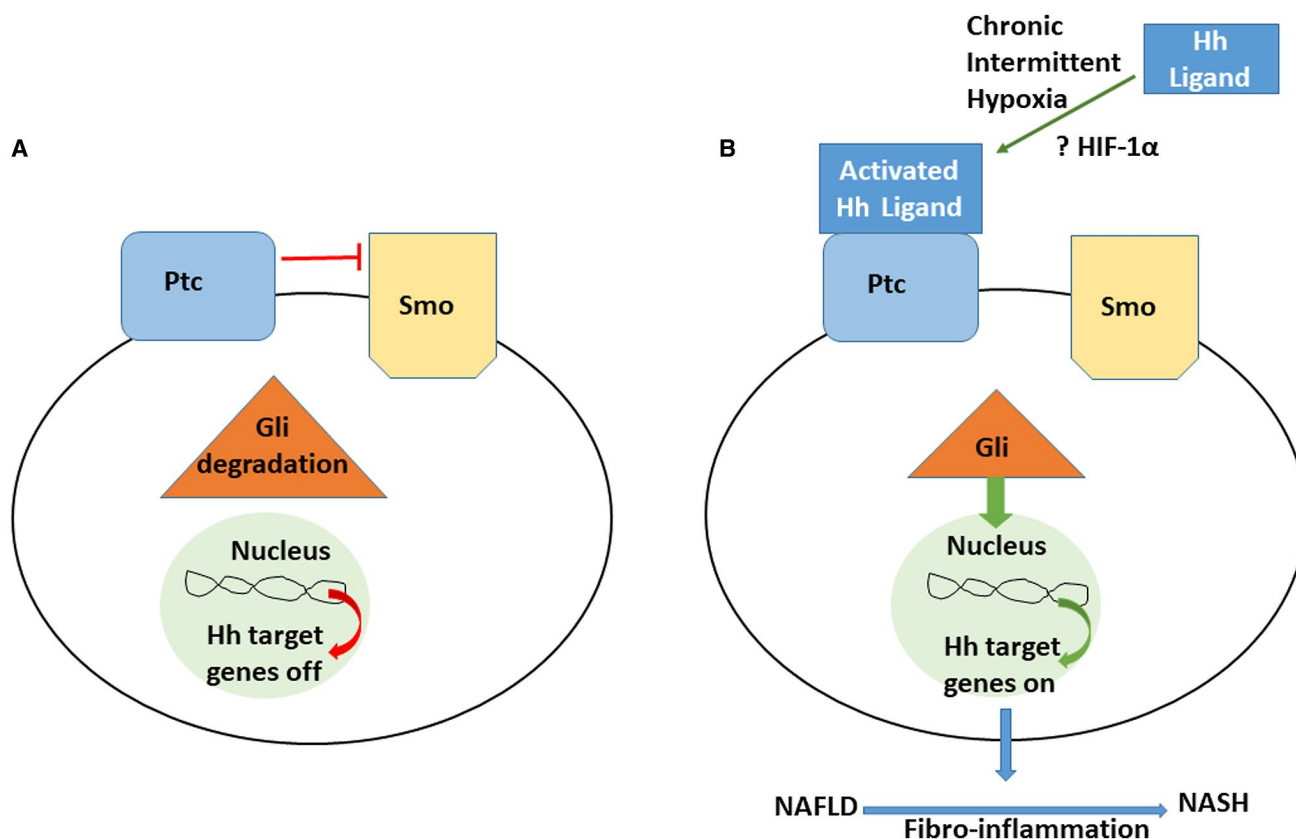
Hypoxia is a known driver for induction of the Hh pathway in several other disease models. Hypoxia induces up-regulation of the Hh pathway, including SHh, Smo, and Gli1, thereby contributing to the invasiveness of pancreatic, breast, and prostate cancer and neuroblastoma.<sup>(29-33)</sup> Cultured cardiomyoblasts exposed to hypoxia also show evidence of induced gene expression of SHh.<sup>(34)</sup> SHh signaling also controls human pulmonary arterial smooth muscle cell proliferation in response to hypoxia.<sup>(35)</sup> Similarly, SHh expression is increased in mouse neurons and neuroprogenitor cells under hypoxic conditions.<sup>(36)</sup> Children with NAFLD have been shown to produce Hh ligand in the progenitor compartment along with accumulation of Hh-responsive (Gli2-positive) cells.<sup>(27)</sup> We now demonstrate that adolescents with NAFLD and OSA/hypoxia also produce Hh in this progenitor compartment together with accumulation of Hh-responsive (Gli2-positive) cells. Taken together, these findings suggest that CIH may have a significant impact on the Hh pathway and consequent hepatic fibro-inflammatory repair response in pediatric NAFLD.

We now show *in vivo* in pediatric NAFLD that the Hh pathway is activated in the liver by CIH. Children with NAFLD and OSA/hypoxia have higher levels of hepatic SHh, Gli2, and  $\alpha$ -SMA than those without OSA/hypoxia. The severity of their OSA correlates strongly with activation of the Hh pathway in the liver, including SHh and Gli2. Both the severity and duration of their hypoxia were also associated strongly with activation of the Hh pathway. Furthermore, children with OSA and hypoxia have more severe fibrosis than those without OSA/hypoxia. Future studies in larger cohorts of children with NAFLD and OSA/hypoxia may further clarify the role of hypoxia-driven activation of the Hh pathways in NAFLD disease progression.<sup>(9,10)</sup>

In OSA and CIH, the liver experiences repeated episodes of nocturnal hypoxia, during which cells adapt to a lower than normal oxygen environment, and normoxia, with sudden increases in oxygen consumption and enhanced mitochondrial generation of

reactive oxygen species.<sup>(37,38)</sup> Tissue hypoxia may allow for functional activation of HIF-1 $\alpha$ , with subsequent induction of genes important in epithelial-mesenchymal transition, including SHh, thereby contributing to NAFLD disease progression. Mice exposed to CIH exhibit increased SHh protein in whole liver homogenates but require HIF-1 $\alpha$  accumulation to mount this response.<sup>(15)</sup> Furthermore, animal models show that HIF-1 $\alpha$  is a proximal effector of Hh-initiated signals that promote glycolysis-dependent changes in hepatic stellate cells.<sup>(39)</sup> Although our HIF-1 $\alpha$  immunohistochemistry did not demonstrate an increase in HIF-1 $\alpha$  protein, a potential role for HIF-1 $\alpha$  in up-regulating Hh signaling in NAFLD remains a biologically plausible possibility. Given that the subjects with NAFLD evaluated in this study had intermittent nocturnal hypoxia and that liver biopsies were taken during the day, transient changes in HIF-1 $\alpha$  may not have been detected because although HIF-1 $\alpha$  responds rapidly to hypoxia, it is also rapidly degraded under normoxic conditions.<sup>(40,41)</sup> In addition, because the intermittent hypoxia was chronic in nature, it is also possible that adaptive changes in HIF-1  $\alpha$  may have prevented its up-regulation and that another hypoxia-induced pathway was responsible for activation of Hh signaling. In the future, studies specifically designed to evaluate HIF-1 $\alpha$  accumulation during nocturnal conditions may help clarify its role.

Limitations of this study include the cross-sectional study design, which precludes us from establishing causality between OSA/hypoxia, the severity of NAFLD, and Hh signaling. The relatively small sample size of this study raises the possibility of type 2 errors in detecting relationships between OSA/hypoxia and Hh signaling. This may explain why, unlike previous work,<sup>(27)</sup> we found strong correlations between aminotransferases, overall NAS score, and Hh markers but not some of the individual components measuring histologic severity. Previous work has suggested changes in Hh signaling with changes in pubertal staging.<sup>(27)</sup> Unfortunately, although all our subjects were Tanner stages 2-4, their specific Tanner stage was not collected and, therefore, we cannot determine the potential impact of this in our study. In addition, our sample may be biased toward more severe liver disease because all enrolled subjects underwent liver biopsy for clinical indications that included chronic elevation of aminotransferases. Finally, our



**FIG. 5.** The fibro-inflammatory response in NAFLD. (A) In the absence of Hh ligands, Ptc represses the activation of Smo, preventing it from interacting with intracellular factors that stabilize and allow for Gli transcription factor accumulation. The Gli proteins subsequently undergo phosphorylation and proteosomal degradation.<sup>(12-14)</sup> (B) The effect of chronic intermittent hypoxia, which we speculate may be mediated by HIF, allows for activation of the Hh ligand, thereby engaging Ptc and preventing Smo inhibition. Activated Smo allows for nuclear localization of Gli transcription factors, which further regulate the expression of Hh-regulated genes that are critically important in the fibro-inflammatory response in NAFLD.

study design does not allow us to specifically examine tissue oxygenation in the liver microenvironment but will be an important aspect of future investigations.

This work contributes to the evolving evidence supporting the dysregulation of the Hh pathway in the pathogenesis of pediatric NAFLD (Fig. 5). CIH, as seen in our study cohort, may allow for activation of the Hh ligand, which we speculate may occur through HIF signaling. The now active Hh ligand triggers the Hh signaling pathway by engaging Ptc, thereby preventing Ptc from inhibiting Smo. Activated Smo allows for the cellular accumulation and nuclear localization of Gli transcription factors, which then regulate the expression of other Hh-regulated genes critical for the proliferation, differentiation, and survival of Hh-responsive cells. Activation of the Hh pathway, a dominant mechanism by which fibro-inflammatory repair occurs, in patients

with NAFLD and OSA/CIH may contribute to both the severity and progression of disease.

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