Second Harmonic Generation Reveals Subtle Fibrosis Differences in Adult and Pediatric Nonalcoholic Fatty Liver Disease

Feng Liu, MD,[1] Jing-Min Zhao, MD,[2] Hui-Ying Rao, MD,[1] Wei-Miao Yu, MD,[3] Wei Zhang, MD,[1] Neil D. Theise, MD,[4] Aileen Wee, MBBS, FRCPath, FRCPA,[5] and Lai Wei, MD[1]

From the [1]Peking University People's Hospital, Peking University Hepatology Institute, Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Diseases, Beijing, China; [2]Department of Pathology and Hepatology, Beijing 302 Hospital, Beijing, China; [3]Institute of Molecular and Cell Biology, Singapore; [4]Department of Pathology, New York University School of Medicine, New York; and [5]Department of Pathology, Yong Loo Lin School of Medicine, National University of Singapore, National University Hospital, Singapore.

Key Words: Automated quantitative analysis; Pediatric; Adult; Liver fibrosis; Nonalcoholic fatty liver disease; Second harmonic generation/two-photon excitation fluorescence; Difference

Am J Clin Pathol December 2017;148:502-512

DOI: 10.1093/AJCP/AQX104

ABSTRACT

Objectives: *Investigate subtle fibrosis similarities and differences in adult and pediatric nonalcoholic fatty liver disease (NAFLD) using second harmonic generation (SHG).*

Methods: *SHG/two-photon excitation fluorescence imaging quantified 100 collagen parameters and determined qFibrosis values by using the nonalcoholic steatohepatitis (NASH) Clinical Research Network (CRN) scoring system in 62 adult and 36 pediatric NAFLD liver specimens.*

Results: *Six distinct parameters identified differences among the NASH CRN stages with high accuracy (area under the curve, 0835-0.982 vs 0.885-0.981, adult and pediatric). All portal region parameters showed similar changes across early stages 0, 1C, and 2, in both groups. Parameter values decreased in adults with progression from stage 1A/B to 2 in the central vein region. In children, aggregated collagen parameters decreased, but nearly all distributed collagen parameters increased from stage 1A/B to 2.*

Conclusions: *SHG analysis accurately reproduces NASH CRN staging in NAFLD, as well as reveals differences and similarities between adult and pediatric collagen deposition not captured by currently available quantitative methods.*

Nonalcoholic fatty liver disease (NAFLD) is a clinical syndrome predicted to be the next global epidemic affecting millions of people worldwide.[1] The prevalence of NAFLD in different regions of China ranges from 6.3% to 27.0% (median, 15%). The prevalence of NAFLD in children in China is 2.1%, although the prevalence increases to 68.2% among obese children.[2] Compared with adults, the prevalence of NAFLD doubled among US adolescents from 3.9% in 1988 to 1994 to 10.7% in 2007 to 2010.[3] The natural course of this disease, including its subtype, nonalcoholic steatohepatitis (NASH), is not clearly defined.[4] Complicating such definition is that while pediatric and adult populations are both affected, the disease is histologically distinctive in each, with differences in the degree of steatosis, qualities and location of histologic steatohepatitis, and patterns of steatofibrosis.[5]

As with the epidemics of hepatitis B and hepatitis C before it, liver biopsy plays an increasing role in documenting the severity of disease in affected individuals, elucidating mechanisms of injury, and evaluating responses to treatment.[5] In the absence of widely effective treatments, the careful monitoring for progression is likely to require, in some patients, repetitive biopsies over time requiring careful comparison. Moreover, evaluation of responses to therapy in clinical trials and, when therapies are standardized, in individual patients will require detailed analysis of histologic features in liver biopsy specimens (LBx), particularly the patterns and degree of scarring and possible features of regression

of fibrosis.[6] For such purposes, semiquantitative staging systems have been developed,[7-11] all of which have pros and cons for any given clinical or investigational setting.[5] Some noninvasive technologies that assess stiffness and/or fibrosis have been developed, including transient elastography, magnetic resonance elastography, and acoustic radiation force impulse imaging, but standards in these tests were developed in adults and may not be applicable to pediatric NAFLD.[12] For some of these tests, steatosis itself is a confounding factor, increasing the stiffness of the liver even in the absence of scar.[13,14] It was found that children with portal-based NASH have more severe fibrosis.[15] Therefore, these noninvasive technologies are inaccurate for fibrosis quantification and insufficient for clinical trials.

Also, compared with patterns of scar in LBx of chronic viral hepatitis, there is a greater variety of patterns, distributions, and locations of scarring in NAFLD LBx. In viral hepatitis, there is a comparatively simple progression from portal fibrosis to portal-portal (sometimes including an intervening vein) fibrous septa, the emergence of parenchymal nodularity, and, finally, advanced/end-stage liver disease ("cirrhosis").[16] On the other hand, in NAFLD, there is considerably more variation: sclerosis of central veins (CVs), stellate fibrosis of portal tracts (PTs), perisinusoidal "chicken wire" fibrosis in varied regions of parenchyma (usually directional from acinus zone 3 to 1), compacted central-central and central-portal fibrous septa, and micronodular to macronodular cirrhosis[6-10]; moreover, the patterns in adults and children are usually different.[5] Thus, the semiquantitative staging systems for viral hepatitis had a somewhat simpler set of patterns to document; the complexity of NAFLD-associated scarring is not entirely captured by currently suggested methods.

Likewise, the relative complexity of NAFLD-associated scar probably reflects a more complex underlying pattern of cellular and molecular events. While most if not all scarring in chronic viral hepatitis depends on mechanisms and patterns of parenchymal extinction,[17] the much more finely fibrillar fibrosis of the classic "chicken wire" perisinusoidal fibrosis of NAFLD is a clearly different process. Furthermore, patterns of regression of fibrosis are also different. While advanced stage viral hepatitis shows a fairly limited set of features, described as the "hepatic repair complex,"[18] patterns and mechanisms of regression of perisinusoidal fibrosis are probably different. These have not yet been greatly detailed in NAFLD. This deficiency is likely to become decisively limiting when it comes to assessment of responses to antifibrotic agents for treatment of NAFLD. Different agents are likely to affect different kinds of scar, from the delicate deposition in the space of Disse to zonally spreading "chicken wire" fibrosis to established fibrous septa. Thus, there is a need for more detailed assessment of changes in scar than is provided by semiquantitative assessments.

Recently, a quantitative approach to detailed assessment of fibrosis has been applied to LBx in chronic viral hepatitis and primary biliary cholangitis.[19-21] Wang et al[22] reported that quantitative fibrosis parameters (q-FPs) could be valid for the assessment of progressing fibrosis in NAFLD by using the system of second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) microscopy. However, the fine detail of collagen distribution in pediatric NAFLD remains unstudied. Therefore, we have applied similar techniques to investigate pediatric NAFLD and compared these findings with the features of adult NAFLD LBx. LBx in NAFLD are imaged by the system of SHG/TPEF microscopy established and adjusted as previously reported.[23] With input of imaging data from the liver sample, quantitative fibrosis (qFibrosis) can automatically compute the fully quantitative fibrosis scores based on the respective collagen architectural features. More important, it can perform in-depth analysis of features such as collagen distribution (eg, aggregated vs distributed), morphology (eg, width, length, perimeter, area, and orientation), and location (eg, portal vs central vs perisinusoidal). Such a strategy potentially overcomes some limitations of semiquantitative histologic fibrosis assessment and can thereby provide a level of detail necessary for fine-grained, thorough assessment of responses to antifibrotic therapies. Here we report the development of NAFLD-qFibrosis, verify its potential as a fibrosis assessment tool, and reveal differences between adult and pediatric patients with NAFLD in a Chinese cohort of patients.

Materials and Methods

Patients and Tissue Preparation

From 2011 to 2015, a total of 98 patients with consecutive biopsy-proven NAFLD from two centers (Peking University People's Hospital and 302 Hospital), comprising 62 adult patients (≥18 years old) and 36 pediatric patients (<18 years old), were included in this study. Patients with liver disease of other etiology, such as alcoholic or drug-induced liver disease, autoimmune liver disease, viral hepatitis, cholestasis, or genetic liver disease were excluded. This study was approved by the Ethical Committee of Human Experimentation in Peking University People's Hospital and 302 Hospital and was

DOI: 10.1093/AJCP/AQX104

performed in agreement with the Helsinki Declaration of 1975. Patients were enrolled after providing written informed consent. LBx were serially sectioned for SHG imaging and stained with H&E, Masson trichrome, and Sirius red for histologic assessment. Clinicopathologic characteristics were obtained from the patients' medical records. Liver fibrosis was staged independently by two pathologists following the NASH Clinical Research Network (CRN) Scoring System,[8] and then discussed before reaching the final consensus. Patterns of steatosis were assessed as described in the NASH CRN study: azonal vs zone 1 vs zone 3 vs panacinar distributions. The fibrosis scores are as follows: 0, none; 1A, mild (delicate) zone 3 perisinusoidal fibrosis, requires Masson trichrome stain to identify; 1B, moderate (dense) zone 3 perisinusoidal fibrosis; 1C, portal fibrosis only; 2, zone 3 perisinusoidal fibrosis with periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis. The minimum length of liver biopsy specimens was 10 mm.

Image Acquisition System

Unstained sections of the tissue samples were imaged using a Genesis (HistoIndex, Singapore) system, in which SHG microscopy was used to visualize collagen, and the other cell structures were visualized with TPEF microscopy.

The samples were laser-excited at 780 nm, SHG signals were recorded at 390 nm, and TPEF signals were recorded at 550 nm. Images were acquired at ×20 with 512 × 512-pixel resolution, and each image had a dimension of 200 × 200 μm^2. Multiple adjacent images were captured to encompass large areas. To cover most of the sample areas, 10 five-by-five multitile images were acquired for each biopsy sample with a final image size of 10 mm^2 (10 × 1 × 1 mm). For comparison, digitized images of liver tissue sections stained by H&E, Masson trichrome, and Sirius red were acquired using light microscopy (B X60; Olympus, Tokyo, Japan).

Image Quantification

One hundred collagen architectural features, including collagen percentages, specific collagen strings, and collagen connectivity-related measurements, were quantified from SHG/TPEF images to assess correlations with the NASH CRN fibrosis stages.

The overall collagen in SHG/TPEF images scanned from the liver tissue sections was divided into three specific regions: CV collagen (CV expansion), PT collagen (portal expansion and bridging fibrosis), and perisinusoidal collagen in the parenchyma. Besides these geographic distributions, collagen measurements were further performed for two different patterns—namely, distributed collagen (fine collagen) and aggregated collagen (large patches). For each pattern, collagen strings were extracted from all regions of collagen and characterized according to length and thickness (long vs short, thick vs thin). Detailed descriptions of all 100 parameters are given in the supplementary materials (all supplemental materials can be found at *American Journal of Clinical Pathology* online).

Statistical Analysis and Model Construction

Continuous variables are expressed as mean ± SD. Categorical variables are summarized by counts and percentages. Comparison of variables was performed using *t* tests for continuous variables and χ^2 tests for categorical variables. Statistical significance was considered when $P < .05$. Spearman's rank correlation and its statistical significance were tested between clinical characteristics and collagen architectural features; the mean and standard error of mean were calculated for each NASH CRN stage for both patient groups.[8] The Wilcoxon rank-sum test was performed to estimate differences in collagen architectural features between different fibrosis stages, as well as between adult and pediatric patients.

For both adult (30 training and 32 validation) and pediatric (18 training and 18 validation) groups, multiple collagen architectural features were used to assess the NASH CRN stages, using a prediction model that was learned with the support vector machine method. The area under curve (AUC) was calculated based on the receiver operating characteristic, and it was used to measure the diagnostic accuracy of the prediction model with the application of NASH CRN stages (S0-S4). Four AUCs were calculated in differentiating (1) patients with S0 and those with S1, S2, S3, and S4; (2) patients with S0 and S1 and those with S2, S3, and S4; (3) patients with S0, S1, and S2 and those with S3 and S4; and (4) patients with S0, S1, S2, and S3 and those with S4, respectively.

Results

Summary of Clinical Data

Clinical data are summarized in Table 1. Sixty-two adult patients, including 35 (56%) women, had a mean age of 43 years (range, 18-76 years). In all, 23 (37%) patients were overweight, and a further 21 (34%) patients were obese. Thirty-six pediatric cases, including six (13%) girls, had a mean age of 12 years (range, 6-17 years) and also were largely overweight (13 [36%] patients) or obese (20 [56%] patients). There were statistical differences among body mass index,[24,25] alanine transaminase, aspartate

Table 1
Clinical Data Summary Comparing Adult and Pediatric Patients With NAFLD[a]

Characteristic	Adult (n = 62)	Pediatric (n = 36)	*P* Value[b]
Age, y	44 ± 16	12 ± 3	<.001
Sex, female vs male	35 (56) vs 27 (44)	6 (13) vs 30 (87)	<.001
Normal BMI[c]	18 (29)	3 (8)	<.001
Overweight	23 (37)	13 (36)	<.001
Obese	21 (34)	20 (56)	<.001
Diabetes mellitus	10 (16)	3 (9)	<.001
Hypertension	6 (10)	0 (0)	<.001
ALT >2 ULN	23 (37)	28 (78)	<.001
AST >2 ULN	11 (18)	18 (50)	<.001
GGT >ULN	27 (44)	17 (47)	<.001
ALP, U/L	88 ± 26	295 ± 90	<.001
Bilirubin, μmol/L	11.5 ± 7.5	10.5 ± 6.5	.503
Serum albumin, g/L	44 ± 7	46 ± 4	.015
Glucose, mmol/L	5.2 ± 1.2	5.0 ± 1.1	.321
Triglycerides, mmol/L	2.8 ± 1.7	1.9 ± 2.0	.025
Platelet count, 10^9/L	219 ± 82	280 ± 68	<.001

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; GGT, γ-glutamyl transferase; ULN, upper limit of normal.
[a]Values are presented as mean ± SD or number (%).
[b]Adult vs pediatric.
[c]Group of China Obesity Task Force[24] and BMI-CHINA Chinese criteria.[25]

Table 2
Pathologic Staging (NASH CRN Scoring System) of Steatofibrosis in Adult and Pediatric NAFLD

Stage	Adult, No. (%)	Pediatric, No. (%)
0	38 (36)	22 (24)
1A	23 (22)	7 (8)
1B	6 (6)	2 (2)
1C	7 (7)	19 (21)
2	6 (6)	19 (21)
3	14 (13)	21 (23)
4	11 (10)	1 (1)
Total	105 (100)	91 (100)

CRN, Clinical Research Network; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

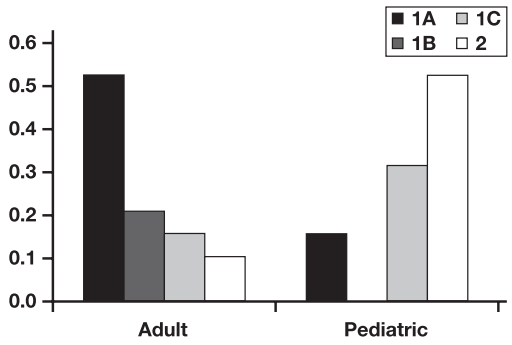

Figure 1 Portal vs central fibrosis in early stage adult and pediatric nonalcoholic fatty liver disease. Most adults had stage 1A fibrosis (ie, predominantly central) while most children displayed stage 1C and 2 disease (ie, predominantly portal, with or without central fibrosis).

transaminase, γ-glutamyl transferase, alkaline phosphatase, and platelet count between adult and pediatric patients with NAFLD ($P < .05$).

Summary of Histologic Findings

Histologic staging of fibrosis in the study LBx is presented in Table 2. In LBx showing early stage NAFLD (stages 1A, 1B, 1C, and 2; ie, when fibrous septa have not distorted liver architecture and thereby obscured landmarks), most adult patients fell into category stage 1A among all stage 1 patients. Only a few patients exhibited stage 2 disease; that is, portal fibrosis was present in only five (26%) of 19 early fibrotic LBx and five (8%) of 62 adult specimens. For pediatric patients, most with early stage NAFLD had some degree of portal fibrosis (stages 1C and 2), comprising 16 (84%) of 19 early fibrotic LBx and 16 (44%) of 36 pediatric specimens Figure 1. According to NASH CRN assessment of patterns of steatosis, in the pediatric group, three patients showed zone 3 steatosis, one patient showed zone 1 steatosis, and the remainder were panacinar.

qFibrosis Assessments

Liver fibrosis progression was quantified using the SHG/TPEF system by examining the collagen levels and morphology. We first investigated the performance of qFibrosis to replicate the fibrosis scores obtained with the NASH CRN system. Patterns of collagen detected by SHG matched the light microscopic appearances and histochemically stained collagen patterns with high fidelity Image 1. Based on their quantitative trends with respect to the fibrosis stages and systemic AUC analyses, six shared parameters mainly for string collagen (StrLength, StrWidth, StrEccentricity, StrSolidity, #StrPT, and #ShortStrPT) were selected from both patient groups; they were demonstrated to identify differences among all fibrosis stages with high AUC (AUC: 0835-0.982 in the adult group, 0.885-0.981 in the pediatric group) Table 3.

Quantitative Features in Portal Tract Collagen Between Adult and Pediatric Groups

The quantitative features in the portal region exhibited similar trends when adult and pediatric LBx were compared, representative highlights of which are shown in Figure 2A and Figure 2B (the remaining data in the

DOI: 10.1093/AJCP/AQX104

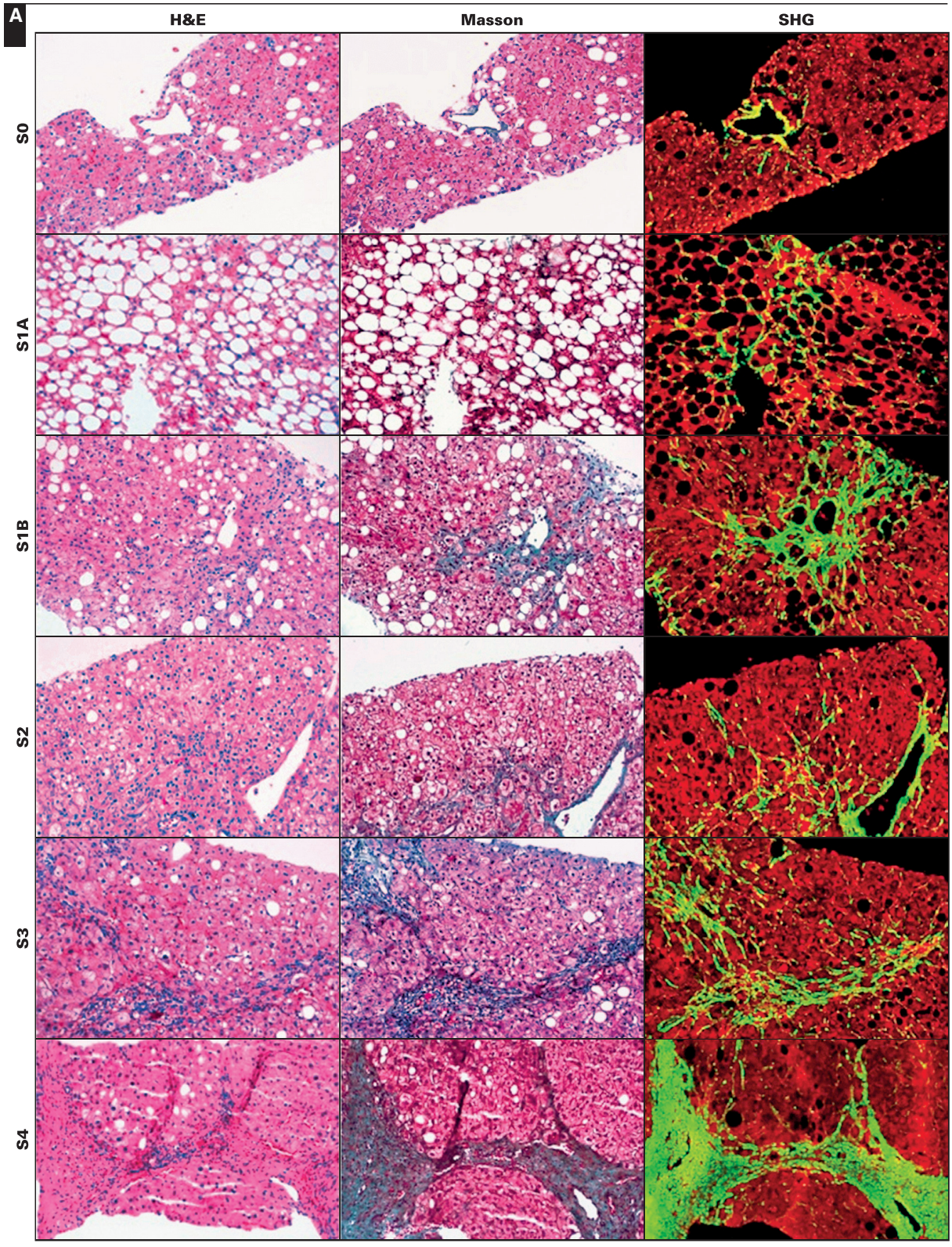

Image 1 Comparison of histopathologic staining (H&E and Masson trichrome) with second harmonic generation (SHG)/two-photon excitation fluorescence images of liver biopsy tissue from (**A**) adult patients with nonalcoholic fatty liver disease (NAFLD).

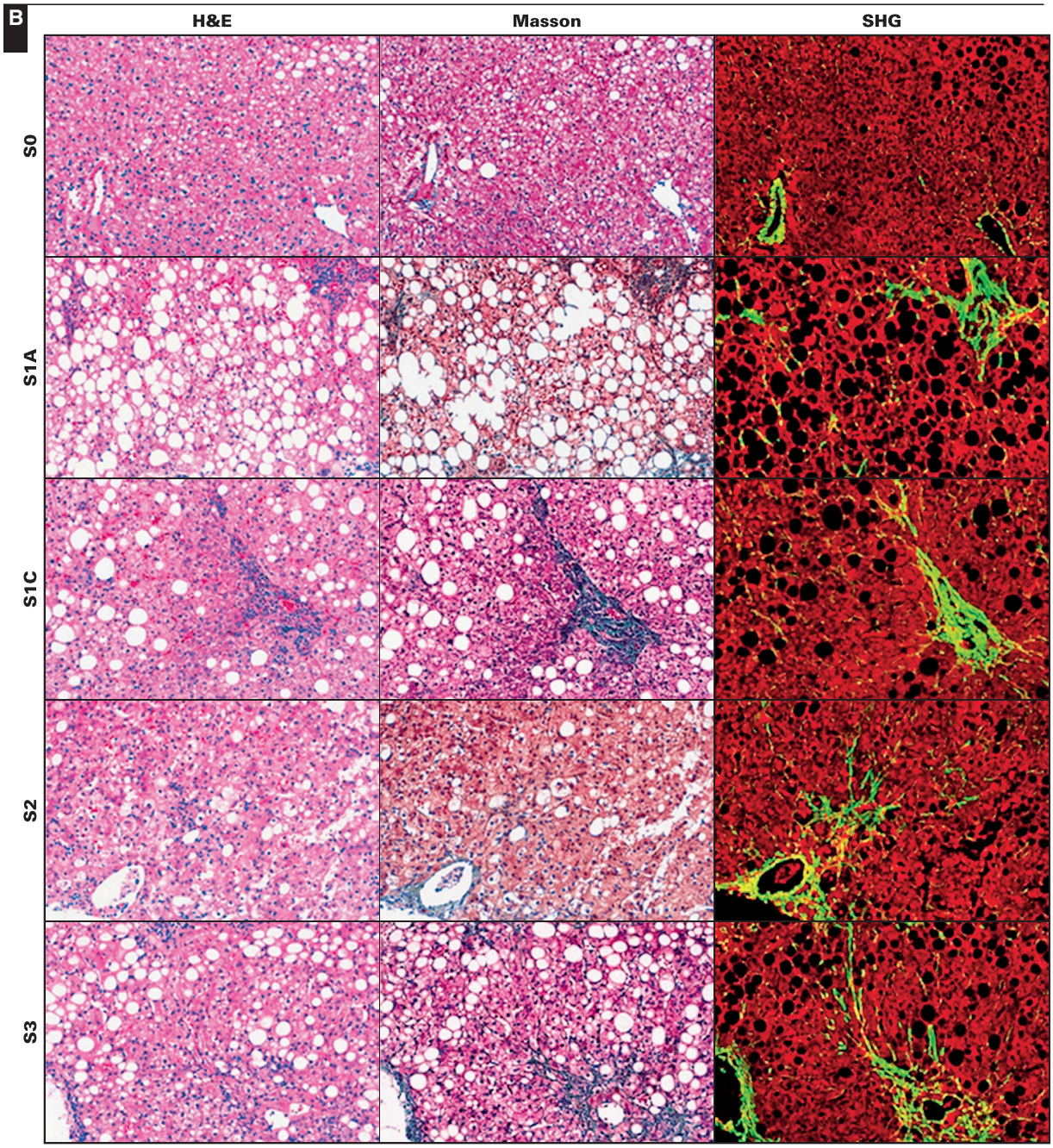

Image 1 (cont) (**B**) pediatric patients with NAFLD. SHG imaging, Masson trichrome, and H&E were performed on serial sections (×200).

supplementary materials). As expected, we found that normal adult PTs showed baseline normal stroma that was greater than normal pediatric PTs, although the differences did not reach statistical significance. All collagen parameters in the portal region showed similar patterns of change when stages 0, 1C, and 2 were compared in both patient groups. In pediatric patients, the portal qFibrosis parameters remained largely unchanged between stages 1C and 2, with the difference between these two stages appearing to be merely the addition of zone 3 collagen without a change in the PTs. In adults, however, the change from stage 1C to 2 seemed to involve a marked increase in PT stroma.

DOI: 10.1093/AJCP/AQX104

Quantitative Features in the CV Collagen Between Adult and Pediatric Groups

Selected representative data concerning changes in CV regions in early stage fibrosis for both patient groups are shown in Figure 2C and Figure 2D (full data presented in the supplementary materials). Adults have higher normal baseline collagen percentage area around CV regions compared with pediatric patients, similar to collagen percentage area around PT regions, but again, not statistically significant. In CV regions, two patterns of change were discernible, rather than the single pattern seen for PT. First, we observed that all parameter values decreased in adults when progressing from stage 1A/B to 2, but in pediatric cases, nearly all aggregated collagen parameters (Figure 2C) decreased and nearly all distributed collagen parameters (Figure 2D) increased from stage 1A/B to 2.

Table 3
Quantitative Fibrosis Assessment of Liver Fibrosis in Adult and Pediatric NAFLD: AUC Values of Six Shared Parameters for String Collagen in Each Fibrosis Stage

Population Group	Stage (NASH CRN Scoring System)	AUC Values (Six Shared Parameters for String Collagen)
Adult	0 vs 1, 2, 3, 4	0.835
	0, 1 vs 2, 3, 4	0.892
	0, 1, 2, vs 3, 4	0.87
	0, 1, 2, 3 vs 4	0.982
Pediatric	0 vs 1, 2, 3	0.981
	0, 1 vs 2, 3	0.931
	0, 1, 2, vs 3	0.885

AUC, area under the curve; CRN, Clinical Research Network; NASH, nonalcoholic steatohepatitis.

Quantitative Features in the Advanced Stages Between Adult and Pediatric Groups

In advanced stages (3 and 4, combined), selected representative data are shown in Figure 3 (remainder of data

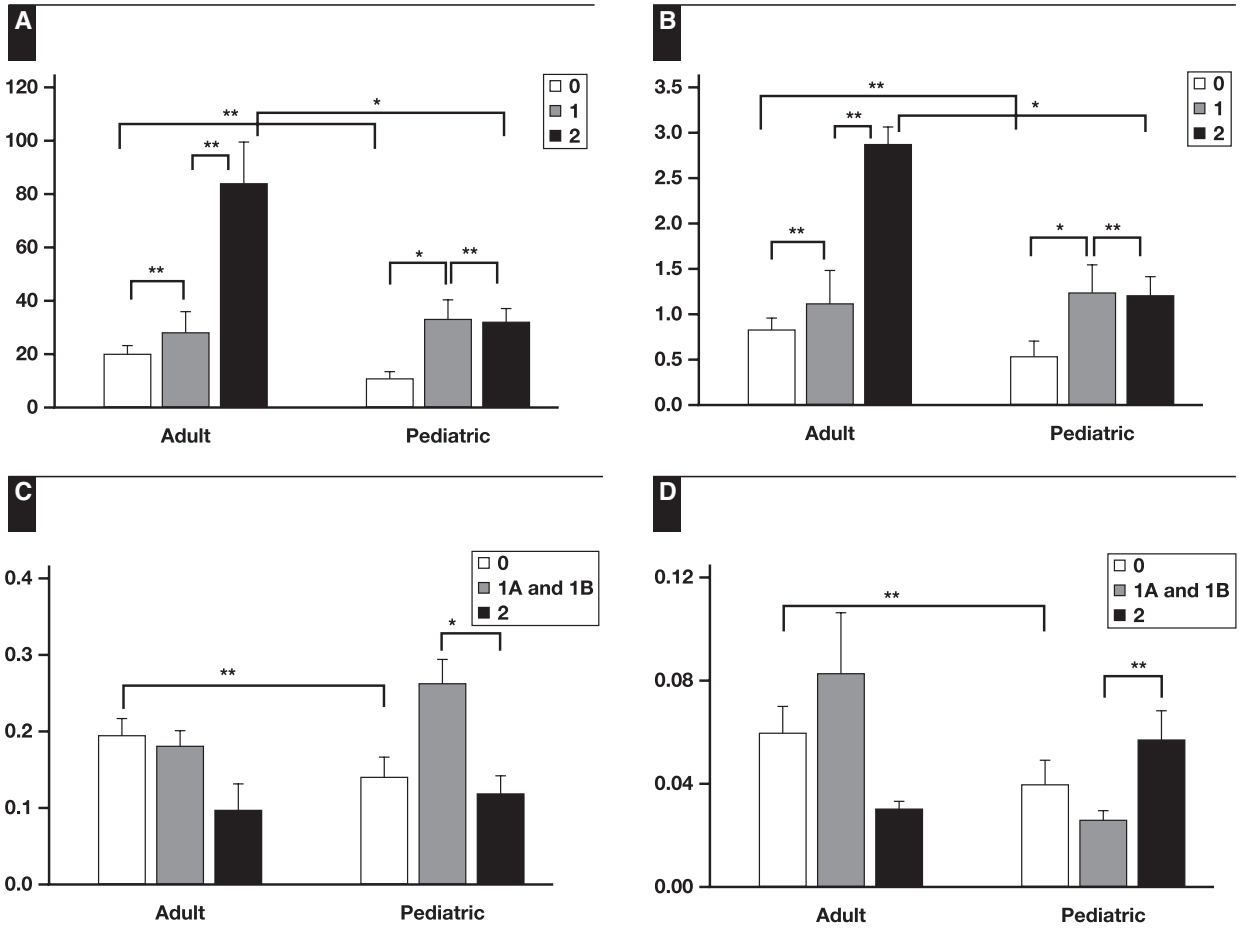

Figure 2 Comparison between adult and pediatric nonalcoholic fatty liver disease of (**A**) the number of collagen intersections in the portal tract region, (**B**) the percentage of aggregated collagen in the portal tract region, (**C**) the number of aggregated long collagen strings in the central vein region, and (**D**) the number of distributed thick collagen strings in the central vein region. *$P < .05$. **$P > .05$.

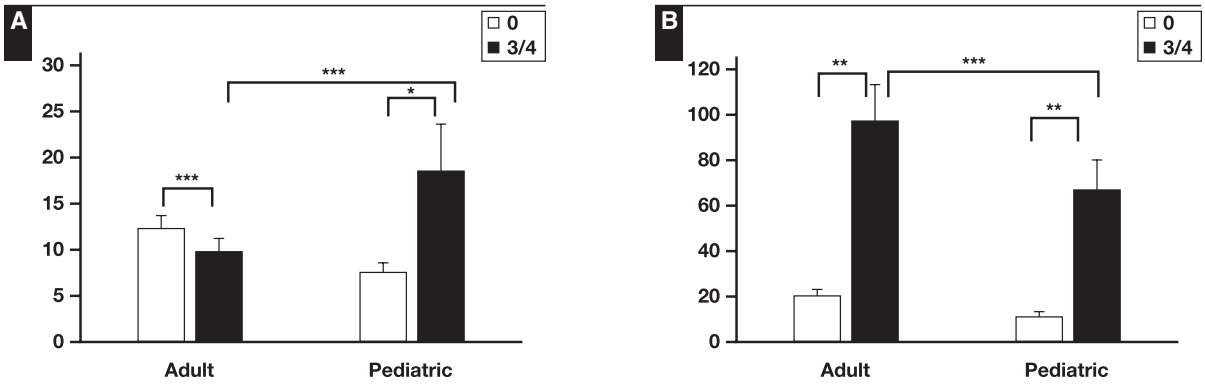

Figure 3 Comparison between adult and pediatric nonalcoholic fatty liver disease of (**A**) the number of collagen intersections in the central vein region and (**B**) the number of collagen intersections in the portal tract region. *$P < .05$. **$P < .001$. ***$P > .05$.

presented in the supplementary materials). Parameters in CV regions were markedly increased in pediatric cases from normal baseline, while adult CV parameters (including size by percent area and all collagen parameters) were largely unchanged (Figure 3A). In PT regions, we observed highly significant increases in PT size (by percent area) and in all collagen parameters for both adult and pediatric groups (Figure 3B). There were no statistical differences for collagen parameter in CV regions and PT regions in the advanced stages between adult and pediatric groups.

Discussion

SHG/TPEF images enabled in-depth analysis of different collagen features, such as collagen distribution (eg, aggregated vs distributed), morphology (eg, width, length, perimeter, area, and orientation), and location (eg, portal vs central vs perisinusoidal). In this study, we developed qFibrosis quantitative assessment based on SHG/TPEF images, which not only accurately reproduces the NASH CRN stages but also reveals subtle differences and similarities between adult and pediatric collagen deposition.

We first showed the feasibility of qFibrosis quantitative assessment in monitoring liver fibrosis progression in both adult and pediatric NAFLD patients by comparing with the NASH CRN staging system. A q-FP method[22] was reported to measure fibrosis stage in adult patients with NASH by using 70 parameters. In our research, six shared parameters selected from 100 parameters, primarily derived from string collagen, were shown to faithfully recapitulate the fibrosis scores. These string collagen parameters and high AUC values may suggest that these parameters have more discriminative power for reflecting the dynamics of fibrosis progression in adult and pediatric patients with NAFLD, and we may be able to identify the patterns for evaluating fibrosis progression in both groups.

Similarly, qFibrosis quantitative assessment not only reliably reflected liver fibrosis staging but also showed the potential to discriminate finer changes between adult and pediatric patients with NAFLD. Several well-known findings are supported by our data. There is slightly more baseline collagen (ie, normal stroma in both PTs and CVs) in adult livers vs pediatric livers.[26] In terms of disease-associated scarring in NAFLD, we confirm the marked predominance of portal fibrosis in pediatric disease compared with the predominance of central fibrosis in adults. Nonetheless, both populations may display features typical of the other, although to a lesser degree: for example, some pediatric LBx show predominantly central fibrosis, while some adult livers show prominent or predominant portal scarring.

qFibrosis quantitative assessment, however, reveals that similarities of staging using the NASH CRN criteria obscure more subtle differences. For example, while adults may sometimes show portal fibrosis superficially similar to that seen in pediatric LBx, the amount of PT collagen is greater in such adult LBx than is present in similarly staged pediatric LBx. Various possible explanations present themselves. Perhaps the lower baseline stroma of pediatric PTs compared with adult PTs means that the percentage change is more similar. Also, steatohepatitis and steatosis are often more prominent in adult LBx,[27,28] raising the possibility of impedance to sinusoidal blood flow in regions where hepatocytes are enlarged, perhaps, thereby, altering the vascular-stromal interplay in portal regions and in turn leading to increased scar. There is also relatively little information regarding the possible

DOI: 10.1093/AJCP/AQX104

roles played by portal fibroblasts compared with sinusoidal hepatic stellate cells (HSCs) in NAFLD, in either age group[29-31]; there may be differences in activation, numbers, and distribution of such different cell populations that are age dependent.

Another interesting finding is the increased size of CV lumens in pediatric compared with adult NAFLD LBx (supplementary results). This finding suggests the possibility of increased hepatic venous outflow, perhaps reflecting increased portal-central vascular shunting. To our knowledge, this has not been previously investigated. While the possible pathophysiologic implications of such a change are unclear, they may merit investigation; for example, increased arterial flow into central areas in pediatric livers may serve to diminish the likelihood of perivenular and central perisinusoidal scarring. It might also signify a compensatory process contributing to the reported lower incidence of stage 4 (cirrhosis) in pediatric patients compared with adults.[32,33]

Also, in CV regions, there were two patterns of change rather than the single pattern observed for PT. Nearly all aggregated collagen parameters decreased and nearly all distributed collagen parameters increased in pediatric cases from stage 1A/B to 2. This "distributed collagen," as described above, represents the fine collagen fibers of the typical perisinusoidal "chicken wire" pattern of steatofibrosis. Thus, there seems to be a difference in the pathophysiology of this kind of scarring between adults and children. One possible mechanism may include differences between HSC activation in pediatric vs adult livers in NAFLD, but to our knowledge, this also remains an unexplored question. In later stages of disease (stages 3 and 4) in which there are clearly defined central-portal and/or central-central fibrous septa, qFibrosis data confirm a generally increased portal scarring in livers of all ages. This convergence on a common pattern of scarring includes most of the individual qFibrosis parameters. We may consider the possibility that at these stages of disease, the pathophysiology of scarring in NAFLD transitions from disease-specific mechanisms involving the sinusoids, the CVs, and the portal regions to a more generalized process of parenchymal extinction, based on vascular occlusions, as proposed by Wanless and Shiota.[34]

In conclusion, as prior independent studies have shown, pediatric and adult patterns of steatofibrosis differ from each other. In pediatric NAFLD, portal fibrosis predominates, while in adult NAFLD, perisinusoidal fibrosis in centrilobular regions predominates, although these patterns are not exclusive of each other. For such general discriminations, semiquantitative staging such as the NASH CRN system suffices; however, we find that more subtle details of the fine, microscopic distribution and structure of collagen are revealed by SHG. We believe that this difference is analogous to the differences in resolution between, for example, the computed tomography scan and magnetic resonance imaging. In this situation, NASH CRN and similar staging systems are primarily assessed at ×2 to ×4, while SHG assesses changes visible at ×40 to ×100 and therefore captures most of the lower power assessment information but adds abundant additional details. Furthermore, the qFibrosis approach, while replicating the semiquantitative staging of the traditional histologic staging, also provides detailed quantification of features identifiable at both low and high magnification.

NAFLD is a multifactorial disease. The pathogenesis of the condition is incompletely understood. The NASH CRN group reported that two-thirds of cases in children with NAFLD show what that study termed the "adult pattern" of steatosis (zone 3) and one-third the "pediatric pattern" (zone 1).[35] However, in our cohort of 36 pediatric patients, we had only three biopsy specimens showing restricted zone 3 steatosis, and one showed the zone 1 pattern; all others showed panlobular steatosis. Also in our research, 16% incidence for 1A fibrosis, 6% for 1B, and 5% incidence for 1C were observed in adult NAFLD. This incidence was similar to 15% incidence for 1A, 6% for 1B, and 6% incidence for 1C reported by the NASH CRN group.[8] Multiple genetic factors of the host and genetic polymorphisms are recognized as significant factors able to modify the individual risk to develop metabolic and inflammatory processes in NASH. Differences between the histologic aspects of our data and those published from the United States may relate, for example, to differences in ethnicity, genetic factors, environmental exposures, and social behaviors (including socioeconomic status, diet, etc). At least 12 genetic polymorphisms have so far been linked with NAFLD, with reported similarities and differences between Asian and non-Asian populations.[36]

We provide here one example of such analysis—namely, the previously developed qFibrosis assessment, which not only accurately reproduces the NASH CRN staging but also reveals differences and similarities between adult and pediatric collagen deposition not captured by semiquantitative methods. We suggest that this higher resolution may be helpful in the development of hypotheses regarding pathophysiologic mechanisms of scarring in different susceptible populations in NAFLD as well as in other diverse diseases. This higher resolution may also be useful for development and testing of novel therapeutic, antifibrotic interventions. One may expect that different classes of antifibrotic agents may affect different types of collagen or different aspects of collagen

production in different sites, by different signaling pathways, and/or by different cell populations. This higher resolution provided by more detailed, quantifiable analysis might thus be useful in preclinical and clinical testing of novel therapeutic agents.

Corresponding authors: Lai Wei, MD, Peking University Hepatology Institute, Peking University People's Hospital Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Diseases, No. 11, Xizhimen South St, Beijing 100044, China; weilai@pkuph.edu.cn. Aileen Wee, MBBS, FRCPath, FRCPA, Department of Pathology, Yong Loo Lin School of Medicine, National University of Singapore, National University Hospital, 5 Lower Kent Ridge Rd, Singapore 119074; aileen_wee@nuhs.edu.sg. Neil D. Theise, MD, Department of Pathology, New York University School of Medicine, New York NY 10016; neil.theise@nyumc.org.

This work was supported by the China-Singapore International S&T Cooperation Program (2016YFE0116800) and grants from the China National Science and Technology Major Project for Infectious Diseases Control during the 12th Five-Year Plan Period (2012ZX10002003 and 2012ZX10002005), the Major Project of National Science and Technology "Creation of Major New Drugs" (2012ZX09303019).

Acknowledgments: We thank Dean C. S. Tai for his constructive comments on technical aspects of this study and Ya-Yun Ren for performing data analysis.

References

1. Sherif ZA, Saeed A, Ghavimi S, et al. Global epidemiology of nonalcoholic fatty liver disease and perspectives on US minority populations. *Dig Dis Sci*. 2016;61:1214-1225.
2. Fan JG. Epidemiology of alcoholic and nonalcoholic fatty liver disease in China. *J Gastroenterol Hepatol*. 2013;28(suppl 1):11-17.
3. Welsh JA, Karpen S, Vos MB. Increasing prevalence of nonalcoholic fatty liver disease among United States adolescents, 1988-1994 to 2007-2010. *J Pediatr*. 2013;162:496-500.e1.
4. Wree A, Broderick L, Canbay A, et al. From NAFLD to NASH to cirrhosis—new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol*. 2013;10:627-636.
5. AlKhater SA. Paediatric non-alcoholic fatty liver disease: an overview. *Obes Rev*. 2015;16:393-405.
6. Brunt EM. Nonalcoholic fatty liver disease: pros and cons of histologic systems of evaluation. *Int J Mol Sci*. 2016;17:E97.
7. Brunt EM, Janney CG, Di Bisceglie AM, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94:2467-2474.
8. Kleiner DE, Brunt EM, Van Natta M, et al; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41:1313-1321.
9. Bedossa P; FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology*. 2014;60:565-575.
10. Bedossa P, Poitou C, Veyrie N, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology*. 2012;56:1751-1759.
11. Alkhouri N, De Vito R, Alisi A, et al. Development and validation of a new histological score for pediatric non-alcoholic fatty liver disease. *J Hepatol*. 2012;57:1312-1318.
12. Crespo M, Lappe S, Feldstein AE, et al. Similarities and differences between pediatric and adult nonalcoholic fatty liver disease. *Metabolism*. 2016;65:1161-1171.
13. Petta S, Maida M, Macaluso FS, et al. The severity of steatosis influences liver stiffness measurement in patients with nonalcoholic fatty liver disease. *Hepatology*. 2015;62:1101-1110.
14. Harris N, Nadebaum D, Christie M, et al. Acoustic radiation force impulse accuracy and the impact of hepatic steatosis on liver fibrosis staging. *J Med Imaging Radiat Oncol*. 2016;60:587-592.
15. Nobili V, Alisi A, Newton KP, et al. Comparison of the phenotype and approach to pediatric vs adult patients with nonalcoholic fatty liver disease. *Gastroenterology*. 2016;150:1798-1810.
16. Quaglia A, Alves VA, Balabaud C, et al; International Liver Pathology Study Group. Role of aetiology in the progression, regression, and parenchymal remodelling of liver disease: implications for liver biopsy interpretation. *Histopathology*. 2016;68:953-967.
17. Wanless IR, Nakashima E, Sherman M. Regression of human cirrhosis: morphologic features and the genesis of incomplete septal cirrhosis. *Arch Pathol Lab Med*. 2000;124:1599-1607.
18. Noureddin M, Anstee QM, Loomba R. Review article: emerging anti-fibrotic therapies in the treatment of non-alcoholic steatohepatitis. *Aliment Pharmacol Ther*. 2016;43:1109-1123.
19. Xu S, Wang Y, Tai DCS, et al. Qfibrosis: a fully-quantitative innovative method incorporating histological features to facilitate accurate fibrosis scoring in animal model and chronic hepatitis B patients. *J Hepatol*. 2014;61:260-269.
20. Wang Y, Hou JL. Current strategies for quantitating fibrosis in liver biopsy. *Chin Med J (Engl)*. 2015;128:252-258.
21. Wu QM, Zhao XY, You H. Quantitative fibrosis parameters highly predict esophageal-gastro varices in primary biliary cirrhosis. *Eur Rev Med Pharmacol Sci*. 2016;20:1037-1043.
22. Wang Y, Vincent R, Yang J, et al. Dual-photon microscopy-based quantitation of fibrosis-related parameters (q-FP) to model disease progression in steatohepatitis. *Hepatology*. 2017;65:1891-1903.
23. Pirhonen J, Arola J, Sädevirta S, et al. Continuous grading of early fibrosis in NAFLD using label-free imaging: a proof-of-concept study. *PLoS One*. 2016;11:e0147804.
24. Group of China Obesity Task Force. Body mass index reference norm for screening overweight and obesity in Chinese children and adolescents [in Chinese]. *Chin J Epidemiol*. 2004;25:97-102.
25. *People's Republic of China Health Industry Standards*.
26. Scheuer PJ. *Liver Biopsy Interpretation (Major Problems in Pathology)*. 4th ed. London, UK: Saunders; 1988.
27. Younossi ZM, Koenig AB, Abdelatif D, et al. Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;64:73-84.
28. Duncan M, Zong W, Biank VF, et al. Nonalcoholic fatty liver disease in pediatrics. *Pediatr Ann*. 2016;45:e54-e58.

DOI: 10.1093/AJCP/AQX104

29. Marcos R, Correia-Gomes C. Long live the liver: immunohistochemical and stereological study of hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells of male and female rats throughout ageing. *Cell Tissue Res*. 2016;366:639-649.
30. Aravinthan AD, Alexander GJ. Senescence in chronic liver disease: is the future in aging? *J Hepatol*. 2016;65:825-834.
31. Grizzi F, Franceschini B, Gagliano N, et al. Mast cell density, hepatic stellate cell activation and TGF-beta1 transcripts in the aging Sprague-Dawley rat during early acute liver injury. *Toxicol Pathol*. 2003;31:173-178.
32. Xanthakos SA, Jenkins TM, Kleiner DE, et al; Teen-LABS Consortium. High prevalence of nonalcoholic fatty liver disease in adolescents undergoing bariatric surgery. *Gastroenterology*. 2015;149:623-34.e8.
33. Patton HM, Lavine JE, Van Natta ML, et al; Nonalcoholic Steatohepatitis Clinical Research Network. Clinical correlates of histopathology in pediatric nonalcoholic steatohepatitis. *Gastroenterology*. 2008;135:1961-1971.e2.
34. Wanless IR, Shiota K. The pathogenesis of nonalcoholic steatohepatitis and other fatty liver diseases: a four-step model including the role of lipid release and hepatic venular obstruction in the progression to cirrhosis. *Semin Liver Dis*. 2004;24:99-106.
35. Africa JA, Behling CA, Brunt EM, et al. In children with nonalcoholic fatty liver disease, zone 1 steatosis is associated with advanced fibrosis [published online March 7, 2017]. *Clin Gastroenterol Hepatol*.
36. Farrell GC, Wong VW, Chitturi S. NAFLD in Asia—as common and important as in the West. *Nat Rev Gastroenterol Hepatol*. 2013;10:307-318.