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Noninvasive Biomarkers in Pediatric Nonalcoholic Fatty Liver Disease

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Abstract

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Nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease worldwide among children and adolescents. It encompasses a spectrum of disease, from its mildest form of isolated steatosis, to nonalcoholic steatohepatitis (NASH) to liver fibrosis and cirrhosis, or end-stage liver disease. The early diagnosis of pediatric NAFLD is crucial in preventing disease progression and in improving outcomes. Currently, liver biopsy is the gold standard for diagnosing NAFLD. However, given its invasive nature, there has been significant interest in developing noninvasive methods that can be used as accurate alternatives. Here, we review noninvasive biomarkers in pediatric NAFLD, focusing primarily on the diagnostic accuracy of various biomarkers as measured by their area under the receiver operating characteristic (AUROC), sensitivity, and specificity. We examine two major approaches to noninvasive biomarkers in children with NAFLD. First, the biological approach that quantifies serological biomarkers. This includes the study of individual circulating molecules as biomarkers as well as the use of composite algorithms derived from combinations of biomarkers. The second is a more physical approach that examines data measured through imaging techniques as noninvasive biomarkers for pediatric NAFLD. Each of these approaches was applied to children with NAFLD, NASH, and NAFLD with fibrosis. Finally, we suggest possible areas for future research based on current gaps in knowledge.

INTRODUCTION

Pediatric nonalcoholic fatty liver disease (NAFLD) is a spectrum of disease that ranges from isolated steatosis, or nonalcoholic fatty liver (NAFL), to its more severe form nonalcoholic steatohepatitis (NASH) (characterized by $\geq 5\%$ hepatic fat infiltration with inflammation and/or hepatocellular ballooning), to fibrosis and even cirrhosis, or end-stage liver disease. It is the most common cause of chronic liver disease in children (1) and has an estimated global prevalence of 5 - 10% (2). In the United States, NAFLD was found to have a prevalence of 9.6% in children (3). This percentage increases markedly in pediatric patients with other metabolic conditions including overweight, obesity, type 2 diabetes mellitus and/or dyslipidemia, with a prevalence of up to 50 - 80% (4,5).

The early identification and management of pediatric NAFLD is crucial in the prevention of disease progression. Alanine aminotransferase (ALT) levels (with upper limit of normal 26 U/L for boys and 22 U/L for girls) are typically used to screen for NAFLD in patients with risk factors (including overweight/obesity, prediabetes/diabetes, features of metabolic syndrome, positive family history of NAFLD) starting at 9 - 11 years of age (6). One study found that using an ALT threshold of twice the upper limit of normal (≥ 50 U/L for boys and ≥ 44 U/L for girls) had a sensitivity of 88% and specificity of 26% for the diagnosis of NAFLD in overweight and obese children (7). In adults, a two-step approach is often used for screening before considering a liver biopsy. This typically involves using a predictive scoring algorithm (e.g. Fibrosis-4 (FIB-4) index) which if elevated, is followed by subsequent imaging (e.g. elastography) to screen for NASH or fibrosis (8,9).

Currently, liver biopsy is considered the most accurate method for diagnosing pediatric NAFLD (6). The Pathology Committee of the NASH Clinical Research Network (CRN) has developed and validated a scoring system that is widely used to assess the severity of NAFLD. This system includes 14 histological features, four of which are evaluated

semi-quantitatively: steatosis (0-3), lobular inflammation (0-3), hepatocellular ballooning (0-2) and fibrosis (0-4). The former 3 features are components of the NAFLD activity score (NAS). A NAS of 5 or higher is indicative of NASH, while scores below 3 suggest simple steatosis, or NAFL (10). Despite its accuracy, given the invasive nature of liver biopsy, associated sampling error, and high cost, there is a significant need for noninvasive techniques to diagnose pediatric NAFLD, NASH, and fibrosis.

NASH is a more advanced and active form of NAFLD⁴⁸ and is characterized by the presence of lobular inflammation and hepatocellular ballooning (11). Individuals with NASH are at an increased risk of liver fibrosis, with progressive scarring that can lead to cirrhosis. Adult⁵¹ studies have demonstrated that liver fibrosis is the most important histologic feature in determining transplant-free survival in adults with NAFLD (12).

This review summarizes the recent research on noninvasive serological biomarkers (serology-based noninvasive tests, NITs), composite scoring algorithms, and imaging biomarkers (imaging-based NITs) used in the diagnosis of pediatric NAFLD, NASH, and NAFLD with fibrosis.

SEROLOGICAL BIOMARKERS / SEROLOGY-BASED NONINVASIVE TESTS

Nonalcoholic Fatty Liver Disease (NAFLD)

Interleukins (ILs)

Several studies have explored interleukins as potential noninvasive biomarkers for the diagnosis of NAFLD in children. Interleukin-1 β (IL-1 β) and IL-6 are secreted by various tissue types, but most abundantly by adipose tissue (13) (**Table 1**). IL-1 β has been implicated in hepatocyte injury and the worsening of NASH (14) while IL-6 is involved in insulin signaling, the synthesis of acute phase proteins, and in regulating chronic inflammation (15). IL-17 is produced primarily by T helper 17 (Th17) cells (16) and functions by linking T cell activation to neutrophil mobilization and activation. IL-17

has been shown to promote the progression of NASH and fibrosis in animal models (17). A recent study found that levels of IL-1 β , IL-6, and IL-17³ were significantly elevated in obese children with NAFLD diagnosed through ultrasound compared to obese controls without NAFLD. These three biomarkers also had excellent diagnostic ability in distinguishing children with obesity and NAFLD from children with obesity without NAFLD. IL-1 β had an area under the receiver operating characteristic (AUROC) of 0.94 (cutoff 11.74 pg/mL, sensitivity 84.6%, specificity 85.2%), IL-6 had an AUROC of 0.94 (cutoff 8.10 pg/mL, sensitivity 91.2%, specificity 80.1%), and IL-17 had an AUROC of 0.97 (cutoff 40.03 pg/mL, sensitivity 89.0%, specificity 93.8%) (18) (Table 2).

Flisiak-Jackiewicz *et al* also evaluated IL-18 as a biomarker of liver steatosis in a cohort of 72 obese children with NAFLD diagnosed through magnetic spectroscopy (MRS). IL-18 is a proinflammatory cytokine associated with metabolic syndrome and hepatocyte injury. It is secreted primarily by macrophages, but also by endothelial cells, vascular smooth muscle cells, Kupffer cells, and adipocytes (19,20). IL-18 was found to be significantly elevated in obese children with NAFLD compared to controls and had¹ an AUROC of 0.68 and a positive predictive value (PPV) of 90% in differentiating between children with or without fatty liver in MRS (21) (cutoff 326.8 pg/mL, sensitivity 60%, specificity 75%). These findings suggest that ILs may be promising serology-based NITs in identifying the presence of NAFLD in children with comorbid obesity.

Adiponectin

Adiponectin is an adipokine with anti-inflammatory and insulin-sensitizing properties. In the liver, adiponectin triggers the peroxisome proliferator-activated receptor- α (PPAR- α) signaling pathway, leading to increased free fatty acid oxidation and reduced gluconeogenesis, giving it antisteatotic properties (22). Studies in adults show that patients with NAFLD have lower levels of adiponectin compared to healthy controls, and that these levels are inversely associated with the degree of hepatic steatosis (23).

Multiple studies have found this correlation to be true in children with NAFLD as well. Boyraz *et al* assessed adiponectin levels in 148 obese children, 63 of whom had liver steatosis diagnosed through ultrasound. The study found lower serum adiponectin levels in obese children with liver steatosis compared to obese controls. Adiponectin was able to differentiate children with advanced liver steatosis from those with mild-moderate steatosis with an AUROC of 0.81 (cutoff 2.56 $\mu\text{g/mL}$, sensitivity 84.21%, specificity 63.64%). In addition, adiponectin was able to differentiate between the presence and absence of NAFLD in obese children with an AUROC of 0.95 (cutoff 3.2 $\mu\text{g/mL}$, sensitivity 100%, specificity 83.53%) (24). Similarly, in a study of 101 obese children with biopsy-proven NAFLD, Mohamed *et al* showed that adiponectin was able to discriminate between NAFLD patients and healthy controls with an AUROC of 0.92 (cutoff value 2.4 $\mu\text{g/mL}$, sensitivity 74.26%, specificity 96.49%) (25). These studies suggest that adiponectin may be utilized in identifying NAFLD in children with and without obesity.

Soluble Adiponectin Receptor 2 (Adipo R2)

While Adipo R2 has been studied in children with NAFLD, there are currently no studies evaluating its diagnostic ability in children with NAFLD. Adipo R2 is abundantly expressed in the liver and skeletal muscle and upon binding adiponectin, mediates fatty acid oxidation and glucose metabolism (26,27). Aksoy *et al* found in a cross-sectional study of 51 obese / overweight children diagnosed with NAFLD through ultrasound that Adipo R2 Levels were higher in obese children with NAFLD as opposed to obese controls. While adiponectin levels were similar in patients with and without NAFLD, this entire cohort of children had adiponectin levels below normal. The authors posit that this lower adiponectin level may have driven a compensatory increase in Adipo R2 expression. Studies have established that decreased hepatic Adipo R2 expression can lead to adiponectin resistance, which can subsequently contribute to NAFLD progression given adiponectin's antisteatotic properties (28). This is the basis behind the study of adiponectin receptor-sensitizing medications, such as

thiazolidinediones, in patients with NASH (29). Further research is needed to validate Adipo R2 as a clinically feasible diagnostic marker of pediatric NAFLD.

Fibroblast Growth Factor 21 (FGF21)

FGF21 is a cytokine secreted primarily by hepatocytes, and to a lesser extent by pancreatic, testicular, duodenal, and adipose tissue (30,31). Liver FGF21 regulates lipid metabolism by promoting lipolysis and reduces hepatic lipid accumulation in an insulin-dependent fashion. Multiple adult studies have found a positive correlation between hepatic steatosis and serum FGF21 Levels (32,33). However, studies in children have been less conclusive. One study found no correlation between FGF21 Levels and NAFLD (34), one found greater FGF21 Levels in obese children with NAFLD (35), and one showed lower FGF21 Levels in children with NAFLD (36). In a study by Alisi *et al* of 84 children with biopsy-proven NAFLD (38% with NASH, 70% with >F0 fibrosis), median levels of FGF21 ⁴⁶ were significantly lower in NAFLD patients compared to controls and serum FGF21 Levels were inversely associated with the probability of NASH and fibrosis (36). A 2016 study in mice reported similar findings, noting that FGF21 knockout mice were more prone to developing NASH (37). A 2019 study in 203 children with steatosis diagnosed through ultrasound found that FGF21 had an AUROC of 0.78 (cutoff 106.10 pg/mL, sensitivity 86.5%, specificity 60%) ² for the prediction of high-grade liver steatosis in the obese and overweight subjects (38). However, this AUROC increased when combined with other biomarkers. Composite algorithms are discussed below in further detail (see “NASH scores” and “Fibrosis scores”).

Resistin and Retinol Binding Protein 4 (RBP4)

Boyraz *et al* also explored resistin and RBP4 as serology-based NITs for pediatric NAFLD. Resistin is a proinflammatory adipokine mainly produced by adipose tissue, inflammatory cells, and hepatic stellate cells (39). RBP4 is a member of the lipocalin family and is primarily expressed in the liver and adipose tissue (40); it acts as a carrier

of retinol in circulation (41). Studies have demonstrated that RBP4 and resistin levels are higher in adults with NAFLD compared to controls (39,42). In differentiating children with advanced steatosis from those with mild-moderate steatosis, resistin had an AUROC of 0.66 (specificity 92.5%) and RBP4 had an AUROC of 0.78 (sensitivity 84.2%). In differentiating children with obesity and NAFLD from controls, resistin and RBP4 had an AUROC of 0.88 (sensitivity 100%) and 0.97 (sensitivity 100%), respectively (24). Further studies in larger cohorts are required to validate the results of this study and establish resistin and RBP4 as clinically feasible biomarkers for children with NAFLD.

Chemerin

Chemerin is an adipokine that enhances insulin-stimulated glucose uptake and insulin sensitivity of adipose tissue (43). It is highly expressed in the liver and adipose tissue, however, its role in NAFLD is unclear (44) and its functional receptor is only expressed on adipocytes and inflammatory cells (45). A prospective case-control study of 101 children with biopsy-proven NAFLD found a significantly higher serum chemerin concentration in obese children with NAFLD compared to non-obese controls. In differentiating obese children with NAFLD from controls, chemerin had an AUROC of 0.78 (cutoff value 186.7 ng/mL, sensitivity 56.44%, specificity 87.72%) (25). Klusek-Oksiuta *et al* also investigated chemerin and found it was able to differentiate children with fatty liver diagnosed through MRS from those without with an AUROC of 0.70 with an optimal cutoff of 190 ng/mL (sensitivity 75%, specificity 58%) (46). While chemerin shows promise as a noninvasive biomarker, it is not a liver-specific adipokine and therefore, its specificity for NAFLD needs to be further investigated.

Visfatin

Visfatin is an adipokine produced by hepatocytes and visceral adipose tissue with a role in glucose and lipid metabolism (44,47,48). While Genc *et al* suggest that visfatin may play a protective role against liver injury in NAFLD, they found no significant

difference in visfatin levels between adults with NAFLD and healthy controls (49). An Iranian study found that children with obesity had higher serum visfatin levels compared to controls, especially when obesity was comorbid with metabolic syndrome or insulin resistance (50). In a study of 80 children with obesity (31 of whom had NAFLD as diagnosed *via* ultrasound), serum visfatin levels were higher in children with dyslipidemia, NAFLD, elevated ALT, fibrosis stage 2 – 3, and steatosis stage 2 – 3. A visfatin cutoff of 18 ng/mL was reported to significantly detect the presence of NAFLD with high sensitivity (83.9%) and specificity (81.4%), making it a promising biomarker for monitoring NAFLD in children with obesity (47).

Nonalcoholic Steatohepatitis (NASH)

NASH is characterized histologically by steatosis, inflammation, and hepatocyte ballooning. Ideal serology-based NITs for NASH would need to highly correlate with these histologic components. This section describes the serological biomarkers that have been investigated thus far in children with NASH (**Table 3**).

Alanine Aminotransferase (ALT)

ALT, synthesized primarily within the cytosol of hepatocytes, is still commonly used in both the clinical setting and in clinical trials as an indicator of liver injury and inflammation. This is largely because ALT is widely available, relatively inexpensive, and requires only a small blood sample (51). Current clinical practice guidelines note that ALT is the best screening test for children with NAFLD and that children older than 10 years with a BMI $\geq 85^{\text{th}}$ percentile should be screened using ALT for NAFLD. In addition, the guidelines state that an ALT > 80 U/L or a persistently elevated ALT greater than twice the upper limit of normal should prompt an evaluation of NAFLD or other causes of chronic hepatitis (6). However, Manco *et al* demonstrated that children with NAFLD may present with normal ALT levels (52) and Molleston *et al* cautioned that ALT levels may underestimate liver injury in children with NAFLD. In their study of children with biopsy-proven NAFLD, children with normal and mildly elevated ALT

showed significant histologic abnormalities including marked steatosis (50% and 24% in patients with mildly elevated and normal ALT, respectively) and advanced fibrosis (stage 3 – 4 in none of the patients with normal ALT, 9% in patients with mildly elevated ALT, 15% in those with elevated ALT). In addition, ALT did not significantly correlate with hepatocyte ballooning, inflammation, or NAS ≥ 4 (53). This raises concerns about the use of ALT in screening children with NAFLD. Interestingly, a recent study by Arsik *et al* evaluated mean ALT over 96 wk as a biomarker for monitoring change in liver histology in children with biopsy-proven NAFLD. Mean ALT was found to be a better predictor of NASH (AUROC 81.84, sensitivity 80.52%, specificity 82.99%) and NASH + fibrosis (AUROC 77.78, sensitivity 71.76%, specificity 80.81%) compared to change in NAS which had a lower AUROC of 0.63. These findings suggest that ALT may be better utilized as a tool for monitoring histologic change in children with NASH and fibrosis longitudinally rather than as a screening tool (54).

Angiopoeitin-2 (Ang-2)

Ang-2 is a potent regulator of vascular development and maturation and is synthesized in the liver, kidney, and endothelial cells. Within the liver, it is produced by liver sinusoidal endothelial cells which, when injured, may promote the progression of simple steatosis to NASH (55). Studies in adults show elevated Ang-2 Levels in patients with histological NASH compared to those with isolated steatosis and that Ang-2 Levels are associated with steatosis, lobular inflammation, and ballooning, but not with fibrosis (56). This finding was reproduced by Manco *et al* who investigated levels of Ang-2 and cytokeratin-18 (CK18), an apoptotic marker, in 76 children with biopsy-proven NAFLD. Ang-2 was elevated in children with NAFLD and NASH compared to controls and was able to predict NASH with an AUROC of 0.911 (cutoff 135.4 ng/mL, sensitivity 85.7%, specificity 85.3%, PPV 83%, negative predictive value (NPV) 87.5%). Ang-2 had a poor predictive ability for differentiating fibrosis from non-fibrosis (AUROC 0.475) (57). Ang-2 appears to be useful in predicting NASH, however, further

research is required to increase the generalizability of the results published by Manco *et al*

Cytokeratin-18 (CK18)

CK18 is a cytoskeletal protein expressed by cells of epithelial origin, including hepatocytes. It is released into the bloodstream during hepatocyte apoptosis as either the whole protein (CK18 M65), which is a measure of total cell death, or the caspase-3-cleaved fragment (CK18 M30), a measure of apoptotic death (58,59). Several studies have evaluated the use of CK18 in adults with NAFLD in predicting NASH with AUROCs ranging from 0.71 to 0.93 (29,60–64). A ⁷ meta-analysis of multiple cross-sectional studies showed that CK18 had a pooled AUROC of 0.82 (median sensitivity 78%, specificity 87%) in predicting NASH in adults with NAFLD (29). A large multicenter study by Feldstein *et al* in 139 adults with biopsy-proven NAFLD found CK18 fragments to have an AUROC of 0.83 (cutoff 279 U/L, sensitivity 71% and specificity 85%) in differentiating NASH from borderline/not NASH (64), further establishing CK18 as a promising biomarker for adult NASH.

Vos *et al* were the first to study CK18 in a pediatric population in a cross-sectional study of 62 children (20 children with obesity and steatosis as diagnosed through ultrasound/CT/elevated ALT >40 U/L; 6 of 20 had biopsy-proven NASH). CK18 Levels were significantly elevated in children with suspected NAFLD compared to obese/normal weight controls and in a multiple regression analysis, had a prediction accuracy of 84.1% for NAFLD (65). Feldstein *et al* studied CK18 in 201 children with biopsy-proven NAFLD (NASH (*n* = 140), no-NASH (*n* = 41)) and found significantly higher CK18 Levels in children with NASH compared to those with isolated steatosis. ⁵ The risk of having NASH on liver biopsy increased with increasing CK18 Levels and CK18 had excellent accuracy in ⁴⁹ predicting the presence of NASH on liver biopsy with an AUROC of 0.933 (cutoff of 233 U/L had sensitivity 85% and specificity 86.9%, PPV 93.7%, NPV 71.6%). This AUROC was significantly higher than those of ALT (AUROC 0.635), AST (AUROC 0.651) or GGT (AUROC 0.672) alone (66). A study of 45 children

with biopsy-proven NAFLD in 2010 found the median value of CK18 M30 was significantly higher in children with NAFLD compared to healthy controls. CK18 M30 had an AUROC of 0.85 in predicting NASH/borderline NASH from simple steatosis in patients with NAFLD (cutoff 207 IU/L, sensitivity 84%, specificity of 88%, PPV 90%, NPV 80%) (67).

In a cross-sectional study of 117 children with biopsy-proven NAFLD, ⁷ greater decreases in serum CK18 Levels were observed in children with histologic improvements compared to those without improvement at 1 and 2 years from baseline. However, change in ALT was found to be a better indicator of NASH resolution (AUROC 0.84) compared to CK18, which had an AUROC of 0.69 ($P = 0.005$). Neither change in ALT, change in CK18, or change in CK18 + ALT had significantly different ⁷ AUROCs for discriminating a ≥ 1 point decrease in steatosis, lobular inflammation, hepatocyte ballooning, or fibrosis stage (68). Further research is required to determine the usefulness of CK18 Levels in tracking the progression of NASH over time. CK18 as a predictor of pediatric liver fibrosis is discussed in the section on diagnosing liver fibrosis below.

Cathepsin D (CatD)

CatD is a lysosomal protease that is ubiquitously distributed in high concentrations in the liver (69). Thus far, it has only been evaluated as a biomarker for children in one study. Walenbergh et al. evaluated the predictive ability of CatD for hepatic inflammation in 96 children with biopsy-proven NAFLD (NASH ($n = 26$), borderline NASH ($n = 51$), steatosis ($n = 19$)). The study found that plasma CatD was significantly lower in children with liver inflammation compared to those with steatosis, and had a negative correlation with increasing liver inflammation, steatosis, hepatocellular ballooning, and NAS. CatD had a high diagnostic accuracy in differentiating between NASH and steatosis with an AUROC of 0.94 and reached an AUROC of 0.998 when combined with CK18. A cutoff of $<18,445$ pg/mL had a sensitivity of 100% and specificity of 89.5% (PPV 92.9%, NPV 100%). CatD as a solo biomarker was also

superior in differentiating NASH from steatosis in children with NAFLD compared to ALT (AUROC 0.59) and CK18 (AUROC 0.72). Additionally, CatD was able to accurately distinguish borderline NASH from definite NASH (AUROC 0.85), steatosis + borderline NASH from definite NASH (AUROC 0.88) and steatosis from borderline NASH + definite NASH (AUROC 0.81), better than ALT or CK18 could on their own. The combination of CatD with CK18 improved the discriminatory ability in each of the aforementioned categories (70). Interestingly, a second study conducted by Walenbergh et al. in 2016 found that in 248 adults with biopsy-proven NAFLD, patients with NASH had increased levels of CatD compared to healthy controls. Following surgical intervention there was a reduction of plasma CatD compared to baseline. This suggests the presence of distinct pathophysiology between childhood and adulthood NASH (71).

Cytokines

Cytokines have also been studied as serology-based NITs for pediatric NASH. A cross-sectional NASH CRN study found that in 235 children with biopsy-proven NAFLD, certain cytokines were significantly associated with different histologic features of NASH. Children with definite NASH and lobular inflammation were found to have significantly higher levels of total (tPAI1) and activated tissue plasminogen activator 1 (aPAI1). In addition, PAI1 was able to significantly discriminate between borderline / definite NASH, definite NASH, lobular inflammation, and hepatocyte ballooning. IL-8 and soluble IL-2 receptor alpha (sIL- 2R α) were associated with fibrosis severity and with lobular and portal inflammation, respectively (72). These findings suggest that these novel cytokines may be a useful tool in the diagnosis and management of NASH in children. However, more research is needed to validate these results and explore the utility of these biomarkers in clinical practice.

Fibrosis

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Currently, liver biopsy remains the gold standard for diagnosing fibrosis in children with NAFLD. However, there is a growing need for noninvasive, more cost-effective diagnostic methods. There have been many advances in recent years in developing noninvasive techniques for diagnosing liver fibrosis, including serological biomarkers, scores based on basic laboratory tests, and imaging modalities. This section discusses the serological biomarkers used in the diagnosis of liver fibrosis in pediatric NAFLD (Table 4). It is important to note that to date, two milestone studies have identified liver fibrosis as the strongest prognostic factor in predicting long-term outcomes in patients with NAFLD (12,73).

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Fibrosis is most commonly scored based on the Metavir score, which includes 5 histologic categories: F0 (no fibrosis), F1 (portal fibrosis with no septae), F1 (portal fibrosis with few septae), F3 (numerous septae without cirrhosis), and F4 (cirrhosis). Any fibrosis refers to F1 – F4, F2 – F4 are considered significant fibrosis, and F3 – F4 are considered advanced fibrosis (74).

Hyaluronic Acid (HA)

HA is a glycosaminoglycan polymer present in epithelial and connective tissue and constitutes a major component of the extracellular matrix (ECM). HA is abundant in the ECM of the liver. Within the liver, HA is primarily produced by activated hepatic stellate cells and degraded by sinusoidal endothelial cells (75). In adults with NAFLD, HA has emerged as a good predictor of liver fibrosis (76–78). In 2010, Nobili *et al* were the first to evaluate HA as a predictive biomarker in children with NAFLD. This study included 100 children with biopsy-proven NAFLD, 65% of whom had ≥stage 1 Liver fibrosis. The study found that serum HA was a good predictor of the degree of fibrosis in children with NAFLD, with HA ≥1200 ng/mL making the absence of fibrosis (F0) unlikely and HA ≥2100 ng/mL making significant fibrosis (≥F2) highly likely. Serum HA as a diagnostic tool for liver fibrosis had an AUROC of 0.88 for any degree of fibrosis (F1-F4 vs F0) when using a cutoff of ≥1200 ng/mL (PPV 90%, NPV 53%) and an

AUROC of 0.95 for significant fibrosis ($\geq F2+$ vs F0-F1) using a cutoff of 2100 ng/mL (PPV 40%, NPV 91%) (79).

In 2011, Lebensztejn *et al* found that HA was significantly higher in children with biopsy-proven NAFLD who had fibrosis compared to healthy controls. With a cutoff value at 19.1 ng/mL, HA had an AUROC of 0.672 (sensitivity 84%, specificity 55%, PPV 52%, NPV 86%) in differentiating children with NAFLD and fibrosis (F1 – F3) from those without fibrosis (F0). When combined with CK18, the AUROC increased to 0.73 (sensitivity 74%, specificity 79%, PPV 56%, NPV 63%). Notably, 37% (19 of 52) of this cohort of children had fibrosis (F1 – F3) (80). Interestingly, the 2010 study by Fitzpatrick *et al* that evaluated CK18 M30 and leptin as biomarkers in 45 children with biopsy-proven NAFLD did not find HA to be a reliable marker of NASH or fibrosis, despite 51.1% of the cohort having $\geq F2$ (significant fibrosis) (67).

N-terminal type III collagen propeptide (PRO-C3)

Fibrosis is a dynamic process that results from the imbalanced production and degradation of ECM proteins, leading to the continuous release of ECM-related proteins into the serum. ¹⁷ PRO-C3, a neo-epitope pro-peptide of type III collagen formation, has been studied as an independent predictor of the degree of fibrosis in adults with NAFLD (81). One such adult study created a PRO-C3 based fibrosis algorithm, named ADAPT (age, presence of diabetes, PRO-C3, and platelet count), that had an AUROC of 0.86 – 0.87 in identifying patients with NAFLD and advanced fibrosis and superior to other fibrosis algorithms ³⁰ such as the Fibrosis-4 (FIB-4) score, NAFLD Fibrosis Score (NFS), and AST to Platelet Ratio index (APRI) ³² (82). Only one group has studied PRO-C3 as a serological marker of fibrosis in pediatric NAFLD. Cohen *et al*, in a study of 88 children with biopsy-proven NAFLD, found that PRO-C3 Levels were similar between children with NAFLD and healthy controls, but significantly lower in children ≥ 15 years compared to children ≤ 10 years old. Amongst children with NAFLD, ² PRO-C3 Levels were higher in children with advanced fibrosis (Ishak score ≥ 3) compared to children with no/mild fibrosis (Ishak score ≤ 2). However, these associations were not

significant after adjusting for bone remodeling biomarkers, suggesting that PRO-C3² may not be a reliable biomarker for liver fibrosis until late adolescence, as it is influenced by age and pubertal growth (83).

Cytokeratin-18 (CK18)

While CK18 has been extensively studied as a marker of pediatric NASH, there is limited data on it as a marker for pediatric liver fibrosis. Lebensztejn *et al* found that CK18 had an AUROC of 0.666 (cutoff 210 U/L, sensitivity 79%, specificity 60%, PPV 56%, NPV 82%) in differentiating children with fibrosis from those without fibrosis.²⁸ When combined with HA, this AUROC increased to 0.73 (80). In 2010, Fitzpatrick *et al* found that CK18 M30 fragments were significantly higher in children with significant or severe fibrosis (\geq F2) compared to children with no/minimal fibrosis ($<$ F2).⁴¹ CK18 M30 had an AUROC of 0.66 in predicting significant/severe fibrosis (\geq F2) (cutoff 200 IU/L, sensitivity 83%, specificity 40%) (67). A more recent study by Mandelia *et al* of 201 children with biopsy-proven NAFLD (68% of cohort with F1 – F3 fibrosis) found CK18 Levels to be significantly higher in children with F1 – F3 compared to F0. Their study had an AUROC of 0.75 in predicting any fibrosis (F1 – F3), AUROC 0.67 in predicting significant fibrosis (F2 – F3) and AUROC 0.77 in predicting advanced fibrosis (F3). Mandelia *et al* also generated a prediction model for fibrosis F1 – F3 that combined CK18 with waist circumference percentile which reached an AUROC of 0.842 of differentiating any fibrosis (F1 – F3) from no fibrosis (F0).⁵² Using this model, they propose that patients with a score of <35 Likely have no fibrosis (specificity 38%, sensitivity 97%, PPV 76%, NPV 86%) and patients with a score ≥ 82 Likely have fibrosis (sensitivity 88%, specificity 59%, PPV 91%, NPV 51%) (84).

Extracellular Matrix (ECM) Associated Noninvasive Tests

Several ECM biomarkers have been studied in adults with NAFLD as biomarkers of fibrosis, including laminin, chitinase-3-like protein 1 (YKL-40),³⁴ amino-terminal propeptide of type III procollagen (PIIINP), and tissue inhibitor of metalloproteinases 1

(TIMP1). YKL-40 is a glycoprotein with an unknown biological purpose, but it is known to promote growth in fibroblasts, chondrocytes, and synovial cells. During fibrogenesis, YKL-40 is released from the hepatic stellate cells (85). Lebensztejn *et al* are the only group to study laminin and YKL-40 in children with NAFLD and found that while they were significantly higher in NAFLD patients with fibrosis compared to healthy controls, neither correlated with fibrosis stage or were useful in predicting fibrosis in children with biopsy-proven NAFLD (80).

Leptin

Leptin is an adipocyte-derived hormone that ¹³ plays a major role in the regulation of appetite and body fat mass and controls energy balance in the hypo- and normoleptinemic states (86,87). Within the liver, leptin is thought to increase hepatic steatosis, steatohepatitis, and fibrosis (88). Leptin has been established as an essential mediator of fibrosis in response to chronic liver injury. A 2002 study demonstrated that ²⁵ Leptin-deficient mice failed to develop fibrosis during steatohepatitis or in response to ⁴² chronic toxic liver injury (89). Leptin exerts its profibrotic effects by activating Kupffer cells and macrophages and stimulating endothelial cells to secrete transforming growth factor- β (TGF- β) (90). In addition, leptin directly targets hepatic stellate cells through the stimulation of TIMP1 production (91). Leptin also protects hepatic stellate cells from apoptosis, leading to a cycle that exacerbates its profibrotic effects (92,93). Studies in adults with NAFLD have found that leptin levels increase in association with increased severity of hepatic steatosis and degree of fibrosis, especially in patients with a high BMI (94).

⁴⁷ Studies have also been conducted evaluating the role of leptin in pediatric NAFLD. In a study of ⁷² children with biopsy-proven NAFLD (36 children each in the training set and validation set), Manco *et al* found that leptin had an AUROC of 0.796 - 0.833 in predicting a NAS of ≥ 5 . In the training set, a leptin cutoff of ≥ 20.4 ng/mL had sensitivity 54%, specificity 76%, PPV 50%, NPV 79%. A risk score that was developed by combining leptin with tumor necrosis factor α (TNF- α) had an AUROC of 0.964 - 0.985.

The risk score showed high accuracy, with a cutoff of ≥ 13.5 having sensitivity 81%, specificity 92%, PPV 82%, NPV 92% (95). This is currently the only study that has done receiver operating characteristic (ROC) analysis regarding leptin in children with NAFLD. However, other studies have evaluated leptin in pediatric NAFLD with varying results. The most recent study by Brandt *et al* found that in a cross-sectional study of 97 prepubertal children with obesity (34% of whom were diagnosed with hepatic steatosis through ultrasound), circulating leptin levels were negatively correlated with the degree of hepatic steatosis (96). However, Nobili *et al* found that circulating leptin levels positively correlated with degree of hepatic steatosis, ballooning, and NAS (independently of age, BMI and gender) in the same cohort of 72 biopsy-proven NAFLD children studied by Manco *et al* (97). Boyraz *et al* similarly found that leptin levels were higher in obese children with steatosis, but that leptin was unable to differentiate obese children with steatosis from obese children without steatosis (24). A 2010 study by Fitzpatrick *et al* in 45 children with biopsy-proven NAFLD found that leptin was able to accurately predict fibrosis grade but not degree of steatohepatitis. Leptin was able to distinguish (67).

Plasma N-terminal Propeptide of Type III Procollagen (PIIINP)

PIIINP is a peptide released during the processing of procollagen. It was first studied in 1984 and found to be normal or slightly elevated in adults with NAFLD (98). In a cross-sectional case-control study of 55 obese children (50 of whom were diagnosed with NAFLD through ultrasound), a PIIINP cutoff of 8.5 ng/mL yield a sensitivity of 74%, specificity 33% in differentiating cases from controls, suggesting that PIIINP may serve as a marker of hepatic steatosis (99). A study of 172 adults with biopsy-proven NAFLD demonstrated that in patients with F0 – F2 fibrosis, PIIINP had an AUROC of 0.77 – 0.82 in discriminating between NASH and simple steatosis, and in patients with F0 – F3, an AUROC of 0.82 – 0.84. When considering patients with all levels of fibrosis, PIIINP was successful in distinguishing between those ²² with simple steatosis and those with NASH or advanced fibrosis with an AUROC of 0.85 - 0.87 (100). Mosca *et al* are the only study

to date to evaluate PIIINP in a biopsy-proven cohort of 204 children with NAFLD. This study found that children with NASH had higher plasma PIIINP levels compared to children without NASH, and that PIIINP levels correlated with NAS and its constituent components. The risk of NASH and \geq F2 progressively increased with increasing PIIINP levels (for every 3.6 ng/mL increase in PIIINP levels, the likelihood of having \geq F2 increased by \sim 14 fold). PIIINP had an AUROC of 0.737 (sensitivity 62%, specificity 91%, PPV 85%, NPV 75%) in discriminating definite NASH from no/borderline NASH. This is higher than the discriminatory ability of the FIB-4 score or APRI (AUROC 0.6369 and 0.6826, respectively). PIIINP had an AUROC of 0.921 for \geq F2 and 0.993 for F3. A cutoff of >8.89 ng/mL had 84% sensitivity, 94% specificity, 95% PPV, 79% NPV for predicting \geq F2, whereas a cutoff of >13.2 ng/mL yielded 100% sensitivity, 98% specificity, 70% PPV, 100% NPV for predicting the presence of F3. These values were higher than those of FIB-4 (AUROC 0.7412 for \geq F2 and AUROC 0.7687 for F3) and APRI (AUROC 0.7659 for \geq F2 and AUROC 0.8535 for F3) (101).

IMAGING-BASED BIOMARKERS / IMAGING-BASED NONINVASIVE TESTS

Ultrasound-Based Biomarkers

Steatosis (primarily controlled attenuation parameter, or CAP)

The ultrasound-based FibroScan® can evaluate the severity of hepatic steatosis and fibrosis. Its best-known function is based on vibration-controlled transient elastography (VCTE) for fibrosis assessment, which works by sending a low-frequency ultrasound shear wave into the liver and measuring the velocity of the wave as it passes through liver tissue. Firmer tissue results in faster wave propagation. This measurement is converted into a liver stiffness measurement (LSM), expressed in kilopascals (kPa), which is able to assess the level of fibrosis in the liver (102). Moreover, the FibroScan® is also able to assess liver steatosis through a measure called controlled attenuation parameter (CAP), expressed as decibels per meter (dB/m), which measures the amount of attenuation of the ultrasound wave as it passes through liver tissue. Higher values of CAP indicate a greater level of hepatic steatosis (103).

Kwon *et al* evaluated the usefulness of FibroScan® in a Korean cohort of 59 obese children and 47 non-obese controls. The study found that children in the obese group had significantly higher levels of CAP and LSM compared to controls and that LSM had a strong positive correlation with conventional predictive indices for hepatic steatosis and fibrosis, including AST, ALT, and APRI (104). A recent study by Chaidez *et al* found CAP to have outstanding discriminatory ability in differentiating steatosis grade 1 – 3 from grade 0 (AUROC 0.98) (Table 5). A CAP cutoff value of ≥ 259 dB/m had a sensitivity of 94%, specificity of 91%, PPV of 97%, and NPV of 91% for the prediction of steatosis grades 1 – 3 (105).

Yang *et al* evaluated the diagnostic efficacy of LSM in NAFLD and its subtypes, NAFL and NASH, in a cohort of 120 children with obesity. The results showed that LSM had an AUROC of 0.768 (sensitivity 70.5%, specificity 70.7%) for NAFLD, an AUROC of 0.674 (sensitivity 61.4%, specificity 64.5%) for NAFL, and an AUROC of 0.725 (sensitivity 64.7%, specificity 65.0%) for NASH. The study concluded that LSM has diagnostic efficacy for NAFLD and its subtypes in children with obesity with optimal predictive values for NAFLD being LSM >4.65 kPa, for NAFL being LSM >4.95 kPa, and for NASH being LSM >5.15 kPa. It is important to note that LSM is typically used in the evaluation of fibrosis, however, in this study LSM was used for the evaluation of steatosis. This study also evaluated CAP with results showing that CAP had an AUROC of 0.757 (sensitivity 67.20%, specificity 67.20%) for NAFLD, an AUROC of 0.659 (sensitivity 59.10%, specificity 60.50%) for NAFL, and an AUROC of 0.722 (sensitivity 70.60%, specificity 72.80%) for NASH. The study concluded that CAP has diagnostic efficacy for NAFLD and its subtypes in children with obesity with optimal predictive values for NAFLD being CAP >258.00 dB/m, for NAFL being CAP >262.50 dB/m and for NASH being CAP >276.00 dB/m (106).

NASH

Yang *et al* also evaluated the ability of CAP and LSM in predicting NASH in children with obesity. They found that CAP had an AUROC of 0.722 (sensitivity 70.6%,

specificity 72.8%) and LSM had an AUROC of 0.725 (sensitivity 64.7%, specificity 65%) in predicting NASH in children with obesity (**Table 6**). The optimal cutoff points were >276 dB/m and >5.15 kPa for CAP and LSM, respectively (106).

Fibrosis

Transient Elastography (TE) / Vibration Controlled Transient Elastography (VCTE)

TE has shown excellent performance in determining the severity of fibrosis in children with NAFLD in two large studies. In a cohort of 52 children with biopsy-proven NASH, Nobili *et al* demonstrated that TE was able to predict any fibrosis (F1 – F4) with an AUROC of 0.977 (cutoff of 5.1 kPa yielded sensitivity 97%, specificity 91%, PPV 97%, NPV 91%) (**Table 6**). It predicted significant fibrosis (F2 – F4) with an AUROC of 0.992 and at a cutoff of 7.4 kPa, had sensitivity 100%, specificity 92%, PPV 80%, NPV 100%. TE was also able to predict advanced fibrosis (F3 – F4) with an AUROC of 1.000 and at a cutoff of 10.2 kPa, had a sensitivity, specificity, PPV, and NPV all of 100% (107). Alkhouri *et al* evaluated the ability of TE in predicting clinically significant (≥F2) fibrosis in children with NAFLD. In their cohort of 67 children with biopsy-proven NAFLD (10 of whom had F2 – F3 fibrosis), TE demonstrated an AUROC of 1.00 in predicting ≥F2 fibrosis. A cutoff of 8.6 kPa yield 100% accuracy in predicting F0 – F1 fibrosis, obviating the need for liver biopsy, and a cutoff of ≥8.6 kPa had 100% accuracy in predicting F2 – F3 fibrosis, highlighting the need for liver biopsy (108). The ability of LSM to predict fibrosis stage was evaluated in a recent study by Chaidez *et al* by comparing LSM to 4 dichotomized outcomes of the Ishak fibrosis scale: no fibrosis (F0) *vs* any fibrosis (F1-F6), mild fibrosis (F0-F1) *vs* moderate-to-severe fibrosis (F2-F6), mild-to-moderate fibrosis (F0-F2) *vs* severe fibrosis (F3-F6), and mild-to-severe fibrosis (F0-F3) *vs* very severe fibrosis (F4-F6). LSM had the strongest discriminatory ability in comparing mild-to-moderate fibrosis (F0-F2) with severe fibrosis (F3-F6) with an AUROC of 0.7 for the NAFLD group ($n = 116$), 0.77 for the non-NAFLD group ($n = 90$), and 0.73 for all participants ($n = 206$) (105).

Acoustic radiation force impulse (ARFI)

ARFI is a noninvasive ultrasonography technique that uses short, high-intensity acoustic pulses to generate shear waves in the liver. These waves propagate at a speed proportional to the stiffness of the tissue, thus traveling faster as the degree of fibrosis increases. These waves are measured as shear wave velocity (SWV) and provide information about the mechanical qualities of the liver being measured. A systematic review of 7 studies (723 adult patients with NAFLD) showed that ARFI had a diagnostic accuracy of 90% in detecting significant fibrosis (sensitivity 80%, specificity 85%) (109). While there have been studies looking at ARFI in children with fibrosis and chronic liver disease, data on the performance of ARFI specifically in children with NAFLD are limited. One pediatric study found that ARFI values correlated strongly with AST/ALT ratios in obese children. In this study of 54 obese children, 90.7% had ARFI <1.19 m/s (normal), 7.4% had ARFI values between 1.19 and 1.75 m/s, and 1.9% had ARFI >1.75 m/s (110). In another study of 148 school children (33.8% with NAFLD), ARFI values were found to correlate positively with hepatic steatosis grades. ARFI detected significant fibrosis (SWV >1.60 m/s) in 7.5% of children, 6% of whom had a normal or mildly steatotic liver on ultrasound, suggesting that children with normal / mild steatosis on ultrasound may have significant fibrosis (111). In a study of 39 children with various biopsy-proven liver etiologies, a SWV of 2.0 m/s had a sensitivity of 100% in detecting advanced fibrosis (\geq F3) (112). A similar study found that in 52 children with chronic liver disease, ARFI was able to discriminate \geq F1 fibrosis with an AUROC of 0.834 (sensitivity 78.9%, specificity 76.9%), \leq F2 with an AUROC of 0.818 (sensitivity 87.5%, specificity 75%), and F4 with an AUROC of 0.983 (sensitivity 100%, specificity 96.7%) (113). Further research is required to investigate ARFI as an imaging-based NIT in the pediatric NAFLD population.

Magnetic Resonance Imaging (MRI)-Based Biomarkers

Steatosis (MRI-Proton Density Fat Fraction, or MRI-PDFF)

MRI is a noninvasive technique that can be used in the evaluation of hepatic steatosis. Magnetic resonance (MR)-based methods are able to measure hepatic fat as a continuous variable and typically ¹² measure the signal fat fraction (SFF), which is ¹² the fraction of the MR signal attributable to hepatic fat. However, this measure can be affected by numerous confounding variables and is scanner dependent. The PDFF removes these confounders and reflects ¹² the fraction of the liver proton density attributable to hepatic fat. This is a direct measure of hepatic fat content and is a fundamental property of the hepatic tissue (114,115)

Several studies have shown MRI-PDFF to strongly correlate with histology steatosis grade in adults (116–119). A 2015 study in children with biopsy-proven NAFLD found that MRI-PDFF was significantly ⁴³ correlated with steatosis grade and that this correlation was influenced by sex and fibrosis stage. The correlation was stronger in girls compared to boys, and weaker in children with more severe fibrosis (F2 – F4) compared to mild fibrosis (F0 – F1) (120). In 2018, a multicenter study in children with biopsy-proven NAFLD found that MRI-PDFF had a high diagnostic accuracy in predicting both histologic steatosis grade and change in histologic steatosis grade over time. It found that MRI-PDFF could discriminate between ³⁷ grade 1 steatosis and grade 2 – 3 steatosis with an AUROC of 0.87 (at a cutoff of 17.5%, it has a sensitivity of 74%, specificity of 90%, PPV of 97%, and NPV of 41%). It could also discriminate grade 1-2 steatosis from grade 3 steatosis with an AUROC of 0.79 (at a cutoff of 23.3%, it yielded the following: sensitivity 60%, specificity 90%, PPV 88%, NPV 65%). MRI-PDFF was able to classify improvement in steatosis grade with an AUROC of 0.76 (sensitivity 31%, specificity 90%, PPV 75%, NPV 60%), and worsening with an AUROC of 0.83 (sensitivity 40%, specificity 90%, PPV 33%, NPV 92%) (121).

A few studies have also investigated MRI-PDFF in discriminating between the presence and absence of steatosis. In 2016, a study of 27 children with biopsy-proven NASH found that a cutoff of 3.5% allowed MRI-PDFF to differentiate between children with NAFLD and healthy controls with a sensitivity of 89% and specificity of 88% (122). A more recent study including 86 children and adolescents (65 overweight or obese),

further investigated the accuracy of MRI in quantifying liver fat against a reference of MRS. MRI-PDFF predicted the presence of steatosis with an AUROC of 0.981 and at a cutoff of 5.4%, yield a sensitivity of 95% and specificity of 91.4% (123).

Recently, Jia *et al* conducted a meta-analysis that showed that MRI-PDFF was accurately able to diagnose stage 1 – 3 steatosis with a summary sensitivity of 95%, specificity of 92% and hierarchical summary ROC (HSROC) of 0.96. MRI-PDFF was additionally found to be more accurate in assessing steatosis in children compared to TE, which had an HSROC of 0.94 with a sensitivity of 86% and specificity of 88% in differentiating S1 – 3 from S0 (124). The high diagnostic accuracy and noninvasive nature of MRI-PDFF, therefore, make it a powerful tool in the evaluation of hepatic steatosis in children.

NASH

There are currently no MR-based imaging studies evaluating NASH in children. Even in adults, multiparametric MRE has only limited diagnostic accuracy for NASH (125). However, a meta-analysis by Kim *et al* which included 485 patients, 207 of whom had simple steatosis and 278 of whom had NASH found that MRI was able to detect NASH with an AUROC of 0.89 and had a pooled sensitivity of 87.4% and pooled specificity of 74.3% (126). Further research is needed to see whether MR is a feasible technique for diagnosing NASH in children.

Fibrosis (Magnetic Resonance Elastography, or MRE)

MRE is an MRI-based, noninvasive tool that can be used to diagnose fibrosis in patients with NAFLD and NASH. It uses propagating mechanical shear waves to determine the mechanical properties of hepatic tissue (127). These shear waves propagate faster in stiffer tissue and the collected wave data is processed by an inversion algorithm that generates cross-sectional, quantitative depictions of the stiffness of hepatic tissue. Unlike ultrasound-based modalities, which provide localized measurements with limited penetration, MRE is able to provide quantitative maps of large regions of the

abdomen at a greater depth, making results independent of abdominal wall fat deposition (128). Studies have shown that MRE is able to accurately determine liver stiffness and assess fibrosis in adults with liver fibrosis (129,130), with an AUROC of 0.86 in predicting advanced fibrosis (131). However, MRE has not reflected the high accuracy seen in adults in the pediatric population.

In a case-series of 35 children with 8 different biopsy-proven chronic liver diseases, Xanthakos *et al* found that an MRE cutoff of 2.71 kPa had a sensitivity of 88% and specificity of 85% in discriminating F0 – F1 from F2 – F4 fibrosis. MRE had an AUROC of 0.92 in this study for detecting significant fibrosis (132). In a multicenter study of 90 pediatric patients, Schwimmer *et al* evaluated the diagnostic utility of two-dimensional gradient-recalled echo MRE (2D GRE MRE) in conjunction with liver biopsy for fibrosis. The study participants, with a mean age of 13.1 ± 2.4 years and 73% male, underwent MRE within 6 mo of liver biopsy. The study found an AUROC of 0.77–0.79 for detection of any fibrosis (\geq F1) and a cutoff of 3.03–3.05 for detection of advanced fibrosis (\geq F3) with an AUROC of 0.88–0.93, sensitivity of 33.3%–50%, specificity of 91.7–94%, PPV of 28.6–30%, and NPV of 95.2–96.2%. The authors caution that cutoffs validated in adult populations may not be appropriate for interpreting pediatric MRE results (133). Trout *et al* found that MRE had an AUROC of 0.70 for differentiating Ludwig stage 0 – 1 from \geq stage 2 fibrosis (defined as fibrosis with few bridges or septa) (134) in 86 children and young adults with a spectrum of biopsy-proven liver diseases. A cutoff of ≥ 2.27 kPa had sensitivity 68.6% and specificity 74.3% while a cutoff of ≥ 1.67 kPa had sensitivity 35.3%, specificity 91.4%. This study also found an AUROC of 0.90 for discriminating Ludwig stage 0 – 2 from \geq Ludwig stage 3 (numerous bridges or septa). A cutoff of 5.41 kPa had a sensitivity of 64.3% and specificity of 93.1%. The study found that MRE was able to better distinguish between stage 0 – 1 and stage 2 – 4 fibrosis in patients without steatosis compared to patients with steatosis; suggesting that steatosis was playing a confounding effect in children with NAFLD (135). A 2019 study in 69 children with biopsy-proven NAFLD found that

MRE liver stiffness values (based on 2D GRE or 2D spin-echo echo-planar imaging pulse sequence) did not significantly differentiate \geq F2 from (136).

NOVEL COMPOSITE SCORES

While several circulating biomarkers have shown promise in their diagnostic abilities for NAFLD, NASH, and fibrosis, none are currently being used in lieu of liver biopsy in the clinical setting. Multiple studies have demonstrated that novel composite algorithms that combine multiple serological biomarkers can sometimes have higher diagnostic accuracy than their solo components.

NASH SCORES

Manco *et al* showed that a combination of Ang-2 and CK18 was able to predict NASH with a sensitivity of 71.4%, specificity of 100% (PPV 100%, NPV 80.4%), which was superior to Ang-2 or CK18 alone (57) (**Table 7**). Similarly, combining CK18 with CatD was able to discriminate steatosis from NASH with an AUROC of 0.998, compared to CK18 (AUROC 0.72) or CatD (AUROC 0.94) alone (70).

A study by Kwon *et al* looked at the use of bone formation biomarkers in children with NAFLD. Procollagen type 1 amino-terminal propeptide (P1NP) is a protein secreted by the ECM that has been implicated in liver disease in adults (137) and in liver fibrogenesis in animal models (138). P1NP is typically elevated in children and adolescents compared to adults given their increased rate of bone formation. To correct for this, the researchers measured levels of serum osteocalcin, another marker of bone formation, and alkaline phosphatase (ALP), in the study cohort. The study found that the P1NP/osteocalcin ratio alone had a diagnostic capability for evaluating steatohepatitis (early fibrosis) with an AUROC of 0.782 (sensitivity 80.9%, specificity 76.9%). However, the diagnostic capability was higher when the ratio was multiplied by ALT, with an AUROC of 0.939 (sensitivity 83%, specificity 92.3%). Similarly, the P1NP/ALP ratio alone had a diagnostic capability with an AUROC of 0.788 (sensitivity

78.8%, specificity 81.3%), but it also showed better diagnostic capability when multiplied by ALT, with an AUROC of 0.894 (sensitivity 82.6%, specificity 92.9%) (139).

FIBROSIS SCORES

Liver fibrosis has been well-established as the most important determinant for survival in adults with NAFLD (12). As end-stage liver disease (also known as cirrhosis (F4)) exists at the end of the fibrosis staging scale, higher fibrosis stages are more likely to progress to cirrhosis compared to earlier stages. Multiple scoring systems have been developed to determine the level of fibrosis in adults with NAFLD, including the AST/ALT ratio, APRI, FIB-4, and NFS. Studies in the last decade have shown that these scores may not be accurately used in the pediatric population for predicting fibrosis (140,141). There have also been scores that have been developed specifically for children, including the pediatric NAFLD fibrosis index (PNFI) and pediatric NAFLD fibrosis score (PNFS). This section will discuss these simple scoring algorithms and novel composite algorithms for the diagnosis of fibrosis in children with NAFLD (Table 8).

Simple Fibrosis Scores

Pediatric studies have evaluated simple serum tests such as ALT, AST/ALT ratio, APRI, NFS, and FIB-4 for their diagnostic performance in detecting fibrosis. These tests have been well-studied in adult populations. One pediatric study reported poor diagnostic performance in detecting any fibrosis ($\geq F1$) for the AST/ALT ratio (AUROC 0.572), FIB-4 (AUROC 0.547), and NFS (AUROC 0.470). However, all three tests performed better for the detection of significant fibrosis ($\geq F2$): AST/ALT ratio (AUROC 0.585), FIB-4 (AUROC 0.686), and NFS (AUROC 0.554). APRI had the best performance of the surveyed tests, with an AUROC of 0.800 for the detection of any fibrosis ($\geq F1$) and 0.628-0.70 for $\geq F3-4$ in the pediatric NAFLD population (140).

A recent study by Mosca *et al* also evaluated APRI, FIB-4, NFS, and a score called Hepamet in predicting the degree of fibrosis in 286 children with biopsy-proven

NAFLD. The Hepamet fibrosis score (HFS) is based on demographic and laboratory data (including sex, age, +/- diabetes, and serum lab values) and was developed to identify adult patients with NAFLD at risk for advanced fibrosis. HFS was able to discriminate between adults with and without advanced fibrosis with an AUROC 0.85 compared to NFS and FIB-4 with AUROC 0.80 ($P = 0.0001$) (142). The study by Mosca *et al* found that APRI had an AUROC of 0.61 (PPV 62.77%, NPV 52.01%) in identifying >F1 fibrosis in children with NAFLD compared to Hepamet which had an AUROC of 0.778 (PPV 63.24%, NPV 61.29%). NFS and FIB-4 both had poor accuracy for the diagnosis of fibrosis with both having AUROC 0.54 (PPV 62%, NPV 52%). APRI and Hepamet both had an AUROC of 0.74 in identifying the presence of >F2 fibrosis, higher than those for FIB-4 and NFS (AUROC 0.58 – 0.60). Interestingly, PNFI had a higher AUROC for identifying both >F1 (AUROC 0.81) and >F2 (AUROC 0.84) compared to these other scores. PNFI was the best noninvasive biomarker in the pediatric age, however, Hepamet showed promise (143).

FibroScan-aspartate aminotransferase (FAST) Score

The FibroScan-aspartate aminotransferase (FAST) score is calculated using LSM, CAP, and serum AST and is used to predict liver disease severity in adults with NAFLD. In adults, it has been found to be an efficient way to non-invasively identify patients at risk of progressive NASH for clinical trials or treatments (144). Chaidez *et al* found that the FAST score had acceptable discriminatory ability for significant liver disease (NAS ≥ 4 and Ishak ≥ 3) with an AUROC of 0.75. At a cutoff of ≥ 0.67 , it had a sensitivity 89% and specificity 62% (105).

Pediatric NAFLD Fibrosis Index (PNFI)

The PNFI was the first noninvasive fibrosis score created for children. Developed by Nobili *et al* in a study of 136 children with biopsy-proven fibrosis, the PNFI uses age, waist circumference, and triglycerides to determine the degree of fibrosis. The PNFI had an AUROC of 0.85 in predicting liver fibrosis ($\geq F1$) and PNFI ≥ 9 had a PPV of 98.5%

(145). However, the AUROC dropped to 0.41 in predicting $\geq F2$ fibrosis in an external validation of the PNFI in a cohort of Korean children (141). Interestingly, Alkhouri *et al* found that PNFI had an AUROC of 0.747 in predicting the presence of clinically significant fibrosis ($\geq F2$) in children with NAFLD. A cutoff of PNFI >8.2 provided 97% accuracy in predicting early fibrosis (F0 – F1) and could be used to rule out patients with significant fibrosis. When combined with data for TE, the algorithm ⁵ could predict the presence or absence of clinically significant fibrosis in 98% of children with NAFLD (108).

Pediatric NAFLD Fibrosis Score (PNFS)

The PNFS was developed in 2014 by Akhouri *et al* from ³⁶ a cohort of 242 children with biopsy-proven NAFLD. The PNFS uses ALT, alkaline phosphatase, platelet counts and GGT and was found to have ¹ an AUROC of 0.74 for the detection of advanced fibrosis ($\geq F3$). At a cutoff of 26%, PNFS had a sensitivity of 31%, specificity 92%, PPV 41% and NPV 88% (146). This scoring algorithm was found to be superior to APRI, FIB-4 and the NAFLD Activity Score (NAS). PNFS has not been validated by outside groups in the pediatric NAFLD population since being developed.

Enhanced Liver Fibrosis (ELF)

The ELF test was first characterized and validated in a cohort of adults with chronic liver disease ⁶ (147). ELF has been studied extensively in adults and shown to be an excellent marker of fibrosis in adults with chronic liver disease (76). It is calculated using three serum biomarkers: HA, PIIINP, and TIMP1. Interestingly, these three markers have themselves been studied as serological biomarkers of fibrosis in children with NAFLD. In studies of adults with NAFLD/NASH, ELF has been validated and found to have ¹⁶ an AUROC of 0.9 in distinguishing severe fibrosis, 0.82 for moderate fibrosis and 0.76 for no fibrosis (76). In a pediatric study of 112 children with likely NAFLD, ELF was able to accurately predict the stage of fibrosis with an AUROC of 0.92 ¹⁹ for any fibrosis (\geq stage 1), 0.92 for moderate-perisinusoidal fibrosis (\geq stage 1b), 0.90 for

moderate-portal/periportal fibrosis (\geq stage 1c), and 0.99 for advanced fibrosis (\geq stage 3). The ELF test was found to have a high accuracy in predicting any fibrosis, with an AUROC of 0.92 (at an optimal cutoff value of 9.28, it had sensitivity of 88%, specificity of 81%, PPV of 90% and NPV of 77%) (148). A later study by Alkhoury *et al* found a lower optimal cutoff of 8.49 (sensitivity 76.9%, specificity 97%). Interestingly, when PNFI and ELF were combined, they were able to predict the presence or absence of fibrosis in 86.4% of children with NAFLD (149). It is important to note that ELF uses biomarkers that are not commonly available in a blood biochemistry panel, and therefore, potentially is less accessible than other fibrosis scores. Another study by Gawrieh *et al* evaluated the relationship of the ELF score with histology in children from the Treatment of NAFLD In Children (TONIC) trial and found that ELF was significantly associated with severity of fibrosis at baseline and 2 years after treatment. In determining the presence of any fibrosis (\geq F1), ELF has an AUROC of 0.60, for \geq F2 AUROC was 0.70, and for \geq F3 AUROC was 0.79 (150). ELF requires further validation in the pediatric NAFLD population prior to being implemented in clinical practice.

CONCLUSION

Pediatric NAFLD has increased in prevalence over the past decade in conjunction with the obesity epidemic. An early diagnosis of NAFLD, NASH, and fibrosis play a large part in preventing disease progression and tailoring management for patients. Therefore, it is very important that we develop and validate noninvasive methods of diagnosis for children with NAFLD.

Adiponectin, IL-1 β , IL-6, and IL-17 all have strong diagnostic accuracy in identifying NAFLD in children with obesity with AUROC values exceeding 0.90. CK18 is extensively studied in children and appears to be the most promising serology-based NIT for the diagnosis of pediatric NASH. Given that adding CK18 to individual biomarkers, such as Ang-2 or CatD, significantly increased their diagnostic abilities, it stands to reason that further research into composite algorithms including CK18 will

generate productive results. While select serological biomarkers (e.g. PIIINP and HA) have AUROCs > 0.90 in predicting advanced fibrosis, most of these serological markers perform poorly in detecting the presence of mild-moderate fibrosis, highlighting the need for further research in this area.

With regard to imaging, MRI-PDFF was superior to ultrasound-based CAP for the diagnosis of steatosis. While TE has excellent performance in differentiating stages of fibrosis, MRI-based methods that have weaker performance have an advantage when it comes to visualization of the liver both in terms of penetration depth and field of view. This is especially important given the rising number of children who have both NAFLD and (morbid) obesity. Future research should focus particularly on better noninvasive imaging modalities for the diagnosis of pediatric NASH, with excellent performance in diverse pediatric populations.

Currently, clinical practice in adults oftentimes utilizes a two-step screening approach for NAFLD prior to considering liver biopsy. In children with NAFLD, one could consider screening with ALT levels first in children with risk factors and, if above the gender-specific upper limit of normal, proceed to subsequent elastography with fat quantification through MRI-PDFF or ultrasound-based CAP measures. MRI-based methods would be preferred over ultrasound-based techniques in obese patients given the higher success rate. Clinicians would then proceed with liver biopsy if imaging reveals concerning findings and/or if ALT levels are persistently elevated >80 U/L. Further studies are warranted to determine the cost-effectiveness of widespread implementation of elastography in the work-up of pediatric NAFLD.

13%

SIMILARITY INDEX

PRIMARY SOURCES

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| <div style="background-color: red; color: white; text-align: center; width: 40px; height: 40px; line-height: 40px; margin: 0 auto;">1</div> | Bryan R. Chen, Calvin Q. Pan. "Non-invasive assessment of fibrosis and steatosis in pediatric non-alcoholic fatty liver disease", Clinics and Research in Hepatology and Gastroenterology, 2022
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