Pediatric nonalcoholic fatty liver disease: A clinical and laboratory challenge

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Abstract
The true prevalence of pediatric nonalcoholic fatty liver disease (NAFLD) is unknown. Challenges in determining the population prevalence of NAFLD include the type of test (and the reference intervals used to define normal and abnormal), the type of population (general population, hospital series), the demographic characteristics of the population sampled, and the nature of the study design. The natural history of pediatric NAFLD remains uncertain. The issue of when to perform a liver biopsy in children with suspected NAFLD remains controversial. Children with NAFLD but normal alanine aminotransferase are rarely investigated. However, evidence of alterations in glucose metabolism parameters should prompt a better understanding of the natural history of pediatric NAFLD not only in terms of the progression of liver disease but also regarding its potential relationship with other health outcomes such as type 2 diabetes mellitus and cardiovascular disease. This evidence could make liver biopsy mandatory in the majority of cases at risk of progressive and severe hepatic and extrahepatic disease. This conclusion, however, raises the question of the feasibility of liver biopsy assessment in an extremely large at risk population, and of the cost/effectiveness of this policy. There is a considerable, continuous interest in reliable, noninvasive alternatives that will allow the prognosis of pediatric NAFLD to be followed in large community or population-based studies.

Key words: Nonalcoholic fatty liver disease; Children; Insulin resistance; Ultrasound; Magnetic resonance imaging

INTRODUCTION
Over the last 2 decades, the rise in the prevalence rates
of overweight and obesity probably explains the emergence of nonalcoholic fatty liver disease (NAFLD) as the leading cause of liver disease in the pediatric population worldwide[1]. NAFLD is a clinicopathologic condition characterized by abnormal lipid deposition in hepatocytes (steatosis) in the absence of excess alcohol intake and represents a spectrum of liver disease ranging from bland (simple) steatosis to nonalcoholic steatohepatitis (NASH) that may lead to fibrosis and cirrhosis. It is a likely common cause of cryptogenic cirrhosis[2]. Once cirrhosis is present, hepatocellular carcinoma may also develop[3]. At present, the most commonly used noninvasive tests to detect NAFLD include measurement of serum aminotransferases and liver ultrasound. Although these tools are useful for the diagnosis of NAFLD, they lack the sensitivity and specificity to distinguish NASH from simple steatosis and determine the presence of fibrosis. Thus, currently, a liver biopsy remains the only reliable way to identify NASH[4]. Despite several advances, there are limited data on the epidemiology and natural history of the disease in children. This review will outline the current knowledge, recent advances, and challenges regarding the prevalence, pathogenesis, natural history, and diagnosis of pediatric NAFLD.

**PREVALENCE OF PEDIATRIC NAFLD**

The true prevalence of pediatric NAFLD is unknown (Table 1)[4][21]. There are inherent challenges in determining the population prevalence of NAFLD. The first challenge is the kind of test (and the cutoff-points used to define normal and abnormal) used in making diagnosis of NAFLD[21]. Diagnosis requires liver biopsy, which is not feasible in a population-based study. Therefore, most studies use serum aminotransferase elevations as a surrogate marker for fatty liver disease (in conjunction with negative markers for other types of liver disease)[22]. Especially germane to this issue is the common use of serum levels of aminotransferases during routine health care examinations to detect unsuspected liver disease[23]. However, the spectrum of fatty liver appears to encompass a wider range of subjects than identified by elevated serum liver chemistries. Franzese et al[1] demonstrated a lack of agreement between ultrasonography and serum aminotransferase levels in cases of fatty liver. Of the 53% of obese children with fatty liver identified by ultrasound, only 32% had abnormalities in serum aminotransferases. In subjects with more severe steatosis, a higher proportion of abnormalities in serum aminotransferases (56%) existed. These findings suggest that heavy infiltration is required for abnormalities in serum aminotransferases to occur. Previous studies have also demonstrated the insensitivity of serum alanine aminotransferase (ALT) in detecting hepatic fat accumulation in obese children as indicated by fast-gradient echo magnetic resonance imaging (fast-MRI) pulse sequences. Using fast-MRI Fishbein et al[24] first showed the insensitivity of serum aminotransferases to detect low levels (< 18%) of hepatic fat fraction (HFF) in obese children. Burger et al[25] then showed that only 48% of obese children with intrahepatic fat accumulation (as evidenced by HFF 5.5% using fast-MRI) had abnormal ALT levels. Finally, we recently showed that obese children with elevated ALT had a much higher mean level of MRI HFF than obese children with normal serum ALT[26].

On the other hand, the way in which clinical laboratories determine their own upper limits of normal values for aminotransferases is critically important in determining the absence as well as the presence of fatty liver disease[27]. Validated standards are not used to establish upper normal limits for ALT and aspartate aminotransferase (AST)[27]. Instead, laboratories use locally defined reference populations to establish their own reference intervals for these tests. Recently, Newschneider-Tetri and colleagues showed that the primary factor contributing to the widely divergent values of upper limits of normal ALT is related to the characteristics of the cohorts used by individual laboratories to establish their own reference intervals[23]. Among factors contributing to the variability in reference cohorts used to establish upper limits of normal values, obesity could be a major factor. As obesity increases in the general population, such reference populations could increasingly include individuals with unsuspected NAFLD, which would skew the upper reference limit to inappropriately high levels. Thus, laboratories should consider identifying healthy adults as well as children without risk factors for insulin resistance and fatty liver disease when establishing reference groups for testing serum ALT and AST levels[23]. Also, anthropomorphic, clinical, or demographic differences other than obesity could be responsible for the variation between laboratories. Care providers often use multiples of reported upper limits of normal values as criteria for further evaluation of the abnormality by imaging, and even liver biopsy. However, multiplying inaccurate upper limits of normal values only multiplies the errant value created by using the local reference population[23]. As expected, the sensitivity and specificity of aminotransferase measurements in the diagnosis of pediatric NAFLD is not established. Alternative approaches include imaging modalities such as liver ultrasound, although the diagnostic accuracy of this approach has its limitations (see section “Laboratory assessment of NAFLD”).

A further challenge in assessing population prevalence is the selection of a representative population with minimal bias[22]. However, most prevalence studies have been conducted in cohorts of children selected for overweight or obesity, many of whom were referred for medical evaluation of obesity. The prevalence of elevated ALT in obese youth has been reported as 10%-14% in the United States adolescents[29], 24% in Hispanic youth[30], 25% in Italian youth[31], 24% in Japanese youth[23], and 48% in youth with type 2 diabetes[32]. There are also studies using ultrasound to assess the prevalence of suspected fatty liver in obese children. The prevalence of echogenic liver in obese youth has been reported as 77% in Chinese youth[33], 28% in German youth[34], 42%-53% in Italian
PATHOLOGIC ASSESSMENT OF NAFLD

Liver biopsy remains an important tool in the diagnostic process in patients with NAFLD. The distinction between simple hepatic steatosis and potentially progressive NASH can only be made by liver biopsy, which can assess the presence and extent of necroinflammation and fibrosis. Nonetheless, even liver biopsy has important limitations that need to be considered. The basic assumption that the small fragment collected through percutaneous liver biopsy is representative of overall hepatic involvement has been seriously challenged. A needle biopsy sample usually represents around 1/50 000 of the total mass of the liver. In addition, many studies have been published showing considerable sampling variability for the sample size. For example, the sample size for the study by Tomina et al. [19] was 1594, which is comparable to the sample size of 810 for the study by Strauss et al. [17]. However, these studies had different sensitivities and specificities, which may explain the differences in the results. Therefore, a large sample size is necessary to obtain accurate prevalence estimates.

FURTHER CHALLENGES

Further challenges in assessing population prevalence are inherent variables based on the age, sex, race, and ethnicity of the population sampled. Keeping this in mind, there are few population-based prevalence studies of pediatric NAFLD. The National Health and Nutrition Examination Survey (NHANES), cycle III, examined a nationally representative sample of children and adolescents between 1988 and 1994. The sample included 2450 adolescents, ages 12 through 18 yr. Abnormal serum ALT levels were found in 75% of adolescents. Other factors associated with elevated ALT levels included increasing age. Data obtained from 1594 subjects aged 10-19 yr from the Korean National Health and Nutrition Examination Survey, cycle III, examined a nationally representative sample of children and adolescents between 1988 and 1994. The sample included 2450 adolescents, ages 12 through 18 yr. Abnormal serum ALT levels were found in 75% of adolescents. Other factors associated with elevated ALT levels included increasing age. Data obtained from 1594 subjects aged 10-19 yr from the Korean National Health and Nutrition Examination Survey, cycle III, examined a nationally representative sample of children and adolescents between 1988 and 1994.

IBW: ideal body weight; BMI: body mass index; NHANES: national health and nutrition examination survey; ALT: alanine aminotransferase.

Table 1: Studies providing an estimate of nonalcoholic fatty liver disease in the pediatric population

| Authors/year/country | Sample size (n) | Age range (yr) | Clinical characteristics | Criteria | Prevalence (%) |
|----------------------|----------------|---------------|-------------------------|----------|---------------|
| Tominaga et al/1995/Japan[4] | 810 | 4-12 | Population-based (Japan) | Ultrasound echogenicity | 2.60 |
| Strauss et al/2000/USA[2] | 2450 | 12-18 | Population-based (NHANES III) | Elevated ALT (> 30 U/L) | 3.00 |
| Park et al/2005/Korea[3] | 1594 | 10-19 | Population-based (Korean NHANES) | Elevated ALT (> 40 U/L) | 3.20 |
| Schwimmer et al/2006/USA[5] | 742 | 2-19 | Population-based (Autopsy data, San Diego County) | Liver histology with ≥ 5% hepatocytes containing fat | 9.60 |
| Tominaga et al/2009/Japan[6] | 846 | 6-15 | Population-based (Japan) | Ultrasound echogenicity | 4.40 |
| Alavian et al/2009/Iran[7] | 966 | 7-18 | Population-based (Iran) | Ultrasound echogenicity | 7.10 |
| Tazawa et al/1997/Japan[8] | 310 | 6-11 | Obese cohort (% IBW/height over 120) | Elevated ALT (> 30 U/L) | 24.00 |
| Fransen et al/1997/Italy[9] | 72 | 4-15 | Obese cohort (% IBW/height over 120) | Ultrasound echogenicity | 53.00 |
| Guzzaloni et al/2000/Ireland[10] | 375 | 8-16 | Obese cohort (BMI > 2 SD for chronologic age) | Ultrasound echogenicity | 42.00 |
| Chan et al/2004/China[11] | 84 | 7-18 | Obese cohort (BMI > 95th percentile) | Ultrasound echogenicity | 77.00 |
| Flores-Calderon et al/2005/Mexico[12] | 80 | 8-10 | Overweight (> 85th percentile) and obese (> 95th percentile) cohort | Elevated ALT (> 40 U/L) | 42.00 |
| Louthan et al/2005/USA[13] | 181 | 4-17 | Obese cohort (BMI > 95th percentile) | Elevated ALT (> 40 U/L) | 8.00 |
| Quiros-Tejeira et al/2007/USA[14] | 517 | 4-19 | Obese cohort (BMI > 95th percentile) | Elevated ALT (> 97.5th percentile for age-and-sex-specific reference values) | 24.00 |
| Rocha et al/2009/Brazil[15] | 175 | 12-15 | Obese cohort (WC > 75th percentile) | Ultrasound echogenicity | 1.70 |
| Denzer et al/2009/Germany[16] | 532 | 8-19 | Obese cohort (BMI > 90th percentile) | Ultrasound echogenicity | 28.00 |
| Papandreou et al/2009/Greece[17] | 43 | 9-13 | Obese cohort | Ultrasound echogenicity | 42.00 |

IBW: ideal body weight; BMI: body mass index; NHANES: national health and nutrition examination survey; ALT: alanine aminotransferase.
most histological features. Ratziu et al. compared histological findings in 51 patients with NAFLD, each of whom had two samples collected through percutaneous liver biopsy. None of the features examined displayed high levels of agreement. Substantial agreement was only seen for steatosis grade; moderate agreement was seen for hepatocyte ballooning and perisinusoidal fibrosis; and lobular inflammation displayed only slight agreement. Six of 17 patients with bridging fibrosis (35%) in one sample had only mild or no fibrosis in the other and, therefore, could have been under-staged by a single biopsy. Ratziu et al. concluded that histological lesions of NASH were unevenly distributed throughout the liver parenchyma and that sampling error in liver biopsy can, therefore, result in substantial misdiagnosis and staging inaccuracies. Merriman et al., through a careful comparison of paired lobular biopsies in subjects at high risk of NAFLD, have also shown significant sampling variability in NAFLD. In their study, agreement for steatosis was excellent, moderate for fibrosis and only fair for most components of necroinflammation. This variability can have an important impact on the diagnostic performance of liver biopsy specimens, as well as in the staging or grading of hepatic disease. Furthermore, liver biopsy is an invasive procedure, and not suitable for repeated evaluations.

It has been demonstrated that the higher the ALT levels, the higher the risk of NASH. Not surprisingly, children are usually selected for liver biopsy to confirm NAFLD where it appears to be the only explanation for the either persistently or intermittently elevated serum aminotransferases, associated with diffusely hyperrechogenic liver tissue at ultrasound examination. At present, children with NAFLD but normal ALT are rarely investigated or indicated for liver biopsy, and the value of performing a biopsy in this situation is still debated. All patients, including children, within the spectrum of NAFLD should be considered potentially affected, not only by hepatic but also by a multisystemic disease. This suspicion would be even stronger in the presence of elevated insulin resistance, which is a sensitive predictor of both progressive liver disease and severe extrahepatic disease. Diabetics and insulin resistance have been reported as the factors most closely associated with severe liver disease in adults as well as in children with biopsy-confirmed NAFLD but in the absence of ALT abnormalities. Hypertension, one of the main features of the metabolic syndrome, has been reported to be more prevalent in adult patients with NAFLD and normal ALT than in those with increased liver enzymes. The clinician should, therefore, be aware that the metabolic alterations related to steatosis and to adipose tissue-related endocrine dysfunction occur independently of overt liver damage and that even fatty liver per se is frequently associated with extrahepatic manifestations of insulin resistance syndrome. Furthermore, studies using a priori selection based on the exclusion of children with normal ALT levels from liver biopsy will not reflect the true extent of NASH-related liver damage in the general pediatric population. Indeed, it is known that liver enzymes may be within the reference intervals in up to 70% of patients with diagnosed NAFLD and that normal liver enzymes cannot be reliably used as a criterion to argue against the usefulness of performing liver biopsy in at risk patients. This evidence could make liver biopsy mandatory in the majority of cases at risk of progressive and severe hepatic disease, as well as of extrahepatic manifestations, unless accurate, noninvasive tests, which are currently unavailable, prove their efficacy. This conclusion, however, raises the question of the feasibility of liver biopsy assessment in an extremely large at risk population, and of the cost/effectiveness of this policy.

The histological features of NAFLD in children include a wide spectrum of alterations including simple steatosis (macroversicular steatosis, characterized by a single or a few large droplets of fat and displacement of the nucleus, in hepatocytes without inflammation), NASH (macroversicular steatosis in hepatocytes associated with inflammation and fibrosis), and cirrhosis. The distinction between simple hepatic steatosis and potentially progressive NASH can only be made by liver biopsy, which can assess the presence and extent of necroinflammation and fibrosis. The minimum criteria for NASH are: steatosis, with macrovesicular fat greater than microvesicular fat; mixed, mild lobular inflammation with scattered polymorphonuclear leukocytes and mononuclear cells; and ballooning degeneration of hepatocytes that are most apparent near steatotic liver cells. A growing body of evidence suggests that children with NASH frequently show histopathological features that differ from those of adults. A unique histological pattern, type 2 NASH, is reported in pediatrics. In this pattern, inflammation and fibrosis are accentuated in the portal area, in contrast to the perisinusoidal-pericellular injury typically observed in adults with NASH (type 1 NASH). The single-center, retrospective study by Schwimmer et al. performed on 100 pediatric patients with biopsy-proven NAFLD, identified type 1 NASH (characterized by steatosis, ballooning degeneration, and perisinusoidal fibrosis) in 17% of subjects, type 2 NASH (characterized by steatosis, portal inflammation, and portal fibrosis) in 51%, and an overlap in 16% of patients. Boys were significantly more likely to have type 2 NASH and less likely to have type 1 NASH than girls. The NASH type differed significantly by race and ethnicity. Type 1 NASH was more common in white children, whereas type 2 NASH was more common in children of Asian, Native American, and Hispanic ethnicity. In cases of advanced fibrosis, the pattern was that of type 2 NASH. The single-center, prospective study by Nobili et al. performed on 84 children (with unknown history of race and ethnicity) with biopsy-proven NAFLD, identified type 1 NASH in 2.4% of subjects, type 2 NASH in 28.6%, and an overlap in 52.4% of patients. Recently, in the multicenter, retrospective study by Carter-Kent et al. performed on 130 children with NAFLD (52% of patients, Caucasian; 1%, African American; 18%, Asian; and 30%, Hispanic),
overlapping features of both type 1 and type 2 NASH were found in 82% of patients. Thus, it is likely that a spectrum of disease patterns exists in pediatric NASH\(^\text{[31]}\).

**PATHOGENESIS OF NAFLD**

A crucial question is that of the underlying difference between people who deposit fat in their liver and those who do not\(^\text{[5]}\). Similarly, it remains to be determined why some of those who deposit fat go on to develop NAFLD, cirrhosis, and liver failure\(^\text{[53]}\). A “two-hit” theory for the development of NASH has been proposed\(^\text{[53]}\). Hepatic steatosis resulting from obesity and hyperinsulinemia is then followed by a second hit of oxidative stress and lipid peroxidation. However, the linkage between inflammatory changes signified by elevation of ALT and further oxidation stress is still unknown\(^\text{[3]}\).

A genetic predisposition is indisputably present in NAFLD, and one possibility is that genetics influence the observed heterogeneity in the development of these traits. Clinical case series have shown familial clustering of NAFLD\(^\text{[8,3]}\). Recent research on heritability of NAFLD has shown how family members of children with NAFLD should be considered at high risk for NAFLD even in the absence of obesity or increased serum aminotransferase levels\(^\text{[56]}\). Furthermore, there are racial and ethnic differences in the prevalence of NAFLD\(^\text{[58]}\).

**Genetic studies**

A number of genes regulate a wide spectrum of mechanisms involved in NAFLD pathogenesis, including lipid accumulation into the liver, oxidative stress, inflammation, and fibrogenesis. Their expression relates not only to fat accumulation but also to the different mechanisms implicated in disease progression\(^\text{[58]}\). Several polymorphisms capable of increasing the severity of disease have been identified\(^\text{[59]}\). For example, a number of studies have analysed genes implicated in liver fat accumulation, adipoïne/cytokine networks, oxidative stress, and fibrogenesis. The microsomal triglyceride transfer protein (MTP) is a key factor for the transfer of triglycerides to nascent apolipoprotein B, producing very low-density lipoprotein (VLDL) and removing lipid from the hepatocyte. The functional polymorphism 493 G/T in the MTP gene has been linked to the severity of liver disease in NAFLD: GG homozygosity, or carrying a lower MTP activity (which would lead to less triglyceride excretion as VLDL, and greater accumulation of lipid inside the hepatocytes) than the other genotypes, predicted more severe liver histology\(^\text{[60]}\), independently of adipokine and insulin resistance\(^\text{[61]}\). Along the same lines, a recent study showed that adiponectin single-nucleotide polymorphisms 45GT and 276GT were more prevalent in NAFLD patients than in the general population, and independently predicted the severity of liver disease in NASH\(^\text{[62]}\). Similarly, a recent study in NASH patients and healthy volunteers evaluated the distribution of the 1183 T/C polymorphism in the mitochondrial targeting sequence of manganese superoxide dismutase (MnSOD), a potent scavenger localized to mitochondria with a key role in scavenging excessive oxidative stress to hepatocytes in NASH patients. This showed that the T/T genotype frequency was significantly higher in NASH patients in comparison with that in the controls. This results in a decrease of MnSOD capacity to detoxify superoxide anions produced in mitochondria, and, therefore, favours excessive oxidative damage inside hepatocytes and NASH progression\(^\text{[63]}\). Miele et al studied Kruppel-like factor 6, previously identified as a ubiquitous transcription factor and immediate early gene expressed in activated hepatic stellate cells after liver injury\(^\text{[64]}\), and therefore possibly involved in the process of liver fibrogenesis. They found that the wild type gene was associated with the severity of fibrosis in NAFLD livers, independently of age, sex, body mass index (BMI), and blood glucose level\(^\text{[65]}\). They also showed preferential transmission of the wild type Kruppel-like factor 6 to children with fibrotic NAFLD\(^\text{[66]}\). Finally, in a population comprising Hispanic, African American, and European American individuals, Romeo et al\(^\text{[66]}\) demonstrated that an amino acid sequence variant [rs 738409 (G), encoding S4531] in patatin-like phospholipase A3 (PNPLA3), a protein of unknown function, was strongly associated with increased hepatic fat levels [evidenced by proton magnetic resonance spectroscopy (MRS)] and with hepatic inflammation (as shown by release of liver enzymes into the circulation). The allele was most common in Hispanics, the group most susceptible to NAFLD; and hepatic fat content was more than twofold higher in PNPLA3 homozygotes than in noncarriers. Resequencing revealed another allele of PNPLA3 [rs6006460 (T), encoding S4531] that was associated with reduced hepatic fat content in African Americans\(^\text{[67]}\), the group at lowest risk of NAFLD. Thus, variation in PNPLA3 contributes to inter-individual differences in hepatic fat content and susceptibility to NAFLD.

**NATURAL HISTORY OF NAFLD**

Current studies suggest that the rate of progression of NAFLD relates to histological severity\(^\text{[68]}\). There is significant debate about the clinical significance and prognosis of simple or “bland”steatosis. This condition is thought to be readily reversible. Once significant fibrosis is present, however, it is unclear if this can be reversed\(^\text{[68]}\). Changes in fibrosis stage have been specifically evaluated in independent series\(^\text{[68,70]}\). Overall, fibrosis progresses over time, but it may remain stable for some years and may improve spontaneously in some cases\(^\text{[68,71]}\). Increased risk of fibrosis appears to be associated with central obesity, insulin resistance states including diabetes as well as features of the metabolic syndrome, in particular high triglyceride and low HDL levels\(^\text{[67]}\). More advanced stages of NAFLD appear to be associated with older age, higher BMI, diabetes, hypertension, high triglycerides, and/or insulin resistance\(^\text{[67]}\). An AST/ALT ratio > 1 may also indicate more severe disease\(^\text{[66,74]}\). The findings...
from different studies are not completely consistent as to which factors are independently associated, and this may depend on the population studied (patients with elevated liver enzymes vs morbidly obese patients vs subjects in the general population)\[68\]. As fibrosis progresses over time, other features of NAFLD, including steatosis, inflammation and ballooning of hepatocytes, significantly improve or disappear\[71\]. In addition, aminotransferases, when elevated, improve or normalize spontaneously over time despite fibrosis progression\[71\]. Thus, fibrosis severity may be the only biopsy feature useful to predict the long-term prognosis in patients with NAFLD. Furthermore, it is possible that the long-term complications of NAFLD might have been underrecognized and underreported, as the characteristic features of macrovesicular steatosis may disappear in the late stages of the disease, leading to a picture of “bland” cirrhosis, which is frequently described as “cryptogenic”, rather than NAFLD-related cirrhosis\[87\].

Most studies evaluating the long-term prognosis of patients with NAFLD originate from specialized care centers at which adult patients had been selected to undergo liver biopsy\[75-79\]. The retrospective analysis by Matteoni et al\[75\] comparing clinical characteristics and outcomes of 98 adult patients with different types of NAFLD over an average (SD) follow-up of 8.3 (5.4) years, demonstrated that the outcome of cirrhosis and liver-related death was not uniform across the spectrum of NAFLD. Poor outcomes were more common in types 3 and 4 NAFLD (currently designed as NASH)\[75,78\]. This study suggested that histological findings may have prognostic value in patients with NAFLD. Other hospital-based studies of the histological subgroup of NAFLD patients with NASH have documented progression to cirrhosis and hepatocellular carcinoma, but have been limited by the small numbers of adult patients and/or average follow-up of less than 5 years\[75,76,79\]. More recently, the study by Rafiq et al, with a median follow-up period of 18.5 years, has shown that liver-related mortality of the NASH cohort [mean (SD) age, 68.9 (10.8) yr] increased to 17.5% in comparison with only 2.7% in the non-NASH NAFLD cohort [mean (SD) age, 71.7 (11.3) yr]\[80\]. These findings confirm that with longer follow-up periods, more NASH patients die as a result of liver-related disease. It also confirms that most patients with non-NASH (simple steatosis or steatosis with nonspecific inflammation) are not subject to liver-related death. This relatively nonprogressive course of non-NASH NAFLD has been reported by others\[79,81\].

Accurate data are also needed on the extent to which NAFLD causes morbidity and mortality in the general population. There are few population-based studies to determine the long-term prognosis of NAFLD. Using the resources of the Rochester Epidemiology, Project, Adams et al\[82\] conducted a population-based cohort study to examine the natural history of patients [mean (SD) age, 49 (15) yr] diagnosed with NAFLD on the basis of imaging studies or liver biopsy. Mean (SD) follow-up was 7.6 (4.0) years culminating in 3192 persons/years follow-up. Liver-related death was the third most common cause of death in those with NAFLD while it was the thirteenth most common cause of death in the general population. Utilizing data from the NHANES III and the NHANES III Linked Mortality File, Ong et al\[83\] conducted a study to determine the overall and liver-related mortality of NAFLD in the general population including 12822 persons. Liver disease was the third cause of death among persons with NAFLD after cardiovascular disease and malignancy\[83]. This risk was independent of obesity or the presence of diabetes mellitus. On the other hand, liver disease was only the eleventh most common cause of death in persons without liver disease\[83\].

Although NAFLD is very common in the pediatric population, data on the prognosis of NAFLD in children remain scant. Given the large number of children affected, it is imperative that we establish a better understanding of the natural history of pediatric fatty liver in terms of the progression of liver disease\[85\]. Although some series have reported documented cases of cirrhotic stage disease in children\[7,84\] or cases of children with NAFLD who developed cirrhosis in young adulthood\[85,86\], cirrhosis is not considered to be a common component of pediatric NASH. Some Authors have speculated that the delay in presentation of cirrhosis until adulthood may be due to the short duration of the process or to introduction of a cofactor after childhood\[87\]. Notably, cirrhosis is reported, though rarely, in patients with NAFLD associated with pituitary dysfunction\[86,89\]. This liver disease, which is more likely to present with or include hepatopulmonary syndrome\[86,90\], will add to the morbidity and mortality of this patient population. Despite careful monitoring and treatment of endocrine abnormalities, recurrence of NASH after liver transplantation has been documented in two children who had a history of hypothalamic/pituitary dysfunction\[78,89\] and developed decompensated liver disease from NAFLD\[90\].

In contrast, hepatic fibrosis is frequently observed in pediatric NASH. Kinugasa et al\[84\] found, by routine laboratory examination, elevated levels of serum transaminases in 36 (12%) of the 299 obese children studied. Liver biopsies carried out in 11 of the 36 children showed fibrous changes in five patients. One patient, a 15-year-old girl with a long history of obesity, had cirrhosis along with maturity-onset diabetes mellitus and hyperlipidemia. Baldridge et al\[81\] reported on a series of 14 obese children with idiopathic hepatic steatosis, identified by retrospective review of all liver biopsies performed in a tertiary-care pediatric hospital. Thirteen of the 14 children had portal fibrosis, and many also had central sclerosis, central portal bridging, and portal-portal bridging. Rashid and Roberts reported on a series of 24 children who were predominantly obese and who underwent percutaneous liver biopsy; all showed large-droplet steatosis, and many had fibrosis (17% or 71%) of varying severity\[84\]. Fibrosis was moderately severe in seven patients. One additional patient had cirrhosis at diagnosis. In a retrospective study, defining the liver biopsy findings in 100 predominantly
obese children with clinical features consistent with NAFLD. Schwimmer et al.\(^{[98]}\) found that simple steatosis was present in 16% of subjects, and advanced fibrosis in 8%. In the study by Nobili et al.\(^{[40]}\), involving 84 obese/overweight children with elevated aminotransferases and diagnosis of NAFLD confirmed via liver biopsy, increased fibrosis was noted in 49 (58%) patients but was mostly of mild (stage 1) severity, with only 4 (4.7%) patients showing septal fibrosis (stage 3). None of the patients showed cirrhosis-stage disease on liver biopsy.

Feldstein et al.\(^{[91]}\) recently reported the first longitudinal study describing the long-term survival of children with NAFLD who underwent a follow-up for up to 20 years. That study demonstrated that NAFLD in children is a disease of progressive potential. Some children presented with cirrhosis, others progressed to advanced fibrosis or cirrhosis during follow-up, and some developed end-stage liver disease with the consequent need for liver transplantation. Feldstein et al.\(^{[91]}\) also showed that NAFLD in children is associated with significantly shorter long-term survival as compared to the expected survival in the general population of the same age and sex. Children with NAFLD had a 13.8-fold higher risk of dying or requiring liver transplantation than the general population of the same age and sex.\(^{[91]}\)

On the basis of the above information, an important goal must be to identify those children with advanced fibrosis, as well as the ones most likely to progress to end-stage liver disease. It is also imperative that noninvasive means be developed to identify children at greatest risk for progressive disease.\(^{[40]}\) Age, sex, race, ethnicity, and severity of obesity have been reported to be associated with the steatohepatitis pattern types.\(^{[96]}\) Older age has been found to be independently associated with increased liver fibrosis in some series.\(^{[96]}\) However, in other series children with advanced stages of fibrosis tended to be younger than those with lesser degrees of fibrosis,\(^{[40]}\) suggesting that yet unidentified susceptibility genes predispose to the more aggressive course in these children.\(^{[40]}\) Most published reports of pediatric NAFLD have reported males to be affected more commonly.\(^{[41-43]}\)

Sex-related differences have been shown in an animal model of NASH, with male sex associated with more severe and diffuse injury.\(^{[94]}\) In the study by Schwimmer et al.\(^{[98]}\), who tested potential associations of distinct patterns of NASH in children, girls with type 2 NASH were more likely to be prepubertal and, therefore, have a hormone profile more similar to young boys with type 2 NASH, whereas girls with type 1 NASH were more likely to be postmenarcheal and thus have higher estrogen levels. Thus, sex hormones are attractive candidates for mediators of the development of and/or protection from NASH. Beside changes in sex steroid hormones, another potential mediator of disease expression and progression is pubertal development.\(^{[40]}\)

During puberty, plasma insulin levels increase, and insulin sensitivity decreases along with multiple other physical and hormonal changes.\(^{[98]}\) This decrease in sensitivity occurs early in puberty, between Tanner stages I and II, with a nadir at Tanner stage III and recovery by stage V.\(^{[99]}\) A longitudinal study examining 60 children at Tanner stage I (ages 9.2 ± 1.4 yr) as well as after 2.0 ± 0.6 yr, showed that, at follow-up, 29 children remained at Tanner stage I while 31 had progressed to Tanner stage III or IV.\(^{[98]}\) In children remaining at Tanner stage I, there was a slight increase in insulin sensitivity with no significant change in acute insulin response or fasting glucose and insulin.

Pubertal transition from Tanner stage I to Tanner stage III was associated with a 32% reduction in insulin sensitivity, and increase in fasting glucose, insulin, and acute insulin response.\(^{[98]}\) These changes were similar across sex, ethnicity, and obesity. Thus, sex steroid hormones and insulin resistance associated with pubertal development may account for the significance of developmental stage in the onset of fatty liver. In that context, the recent study of Patton et al.\(^{[40]}\) may be of significant interest. These authors found that lower Tanner stage was predictive of higher fibrosis scores, suggesting that hormonal changes associated with pubertal development may influence disease severity.\(^{[40]}\) However, as pointed out by Patton et al.\(^{[40]}\), whether children with borderline zone 1 pattern may evolve into definite NASH and/or children with definite NASH regress to borderline zone 3 or simple steatosis upon further developmental maturation is unknown and will require longitudinal data to determine.

In some recent series, the presence and severity of fibrosis was associated with a higher BMI.\(^{[94,41,43]}\) However, data obtained prospectively from children enrolled in the NASH Clinical Research Network reported no association of BMI with fibrosis severity,\(^{[94]}\) although the percentage of body fat was lower among subjects without fibrosis.\(^{[98]}\) It may be that body fat distribution (or adiposity with a central distribution)\(^{[96]}\) is a more important determinant of fibrosis than BMI. Body fat distribution affects insulin sensitivity and varies by race and ethnicity.\(^{[40]}\) Ellis et al.\(^{[100]}\) showed that, after adjustment for body size, Hispanic children have significantly higher body fat and percentage fat than white or black children. In a study evaluating clinical correlation of histopathology in pediatric NAFLD, Hispanic ethnicity was predictive of fibrosis severity when comparing those with mild and moderate degrees of fibrosis.\(^{[100]}\) In another study aiming to define key differences between the NASH subtypes, the majority of biopsies from children of the white race had type 1 NASH, while type 2 NASH was the major form seen in children of Asian and Native American race and Hispanic ethnicity.\(^{[98]}\) In the same report, biopsies from children of the black race mostly showed simple steatosis.\(^{[98]}\)

Previous studies in adults with NAFLD have shown that components of metabolic syndrome may contribute to severe liver steatosis, NASH activity, fibrosis or isolated portal fibrosis.\(^{[42]}\) It is, therefore, also important that we establish a better understanding of the natural history of pediatric NAFLD in terms of its potential relationship with other health outcomes including type 2 diabetes mellitus and cardiovascular disease.\(^{[7]}\) Studies in children have demonstrated the relationship between
fasting hyperinsulinemia and dyslipidemia\cite{101,108}, hypertension\cite{104,106}, and impaired glucose tolerance\cite{107}. Schwimmer \textit{et al.}\cite{41} first extended these data to include NAFLD in children as being related to fasting hyperinsulinemia and insulin resistance. They showed that, even after subjects with diabetes were excluded, almost all children with biopsy-proven NAFLD had insulin resistance. Portal inflammation was predicted by the combination of ALT and fasting insulin, whileportal fibrosis was indicated by the combination of right upper quadrant pain and homeostasis model assessment of insulin resistance\cite{41}. In another series of pediatric NAFLD, higher insulin levels also were predictive of mild fibrosis\cite{46}. Recently, Manco \textit{et al.}\cite{52} showed that fasting insulin secretion trended to be increased in children with fibrosis compared to those without fibrosis. More recently, Patton \textit{et al.}\cite{127} showed that severity of insulin resistance was the component most consistently associated with histological features of NAFLD, showing significant associations with severity of steatosis, fibrosis, hepatocyte lular ballooning, and NAFLD pattern. Although in clinical practice insulin resistance is unlikely to be of use in distinguishing fibrosis stage, the above findings support insulin resistance as key variable in disease progression\cite{109,111}. Evidence of alterations in glucose metabolism parameters should prompt a careful follow-up of pediatric patients to prevent major complications\cite{44}.

Higher levels of serum AST have been found associated with fibrosis in some series of pediatric NAFLD\cite{42,47}. However, neither AST levels alone nor the combination of routinely available laboratory data have shown sufficient specificity or sensitivity to predict the presence of severe fibrosis in children with NAFLD\cite{45}. Recent data from adult as well as pediatric patients underscore that NAFLD has to be considered a potentially progressive disease even in the presence of normal ALT levels\cite{42,47,53}. In that vein, it is remarkable that in one series of pediatric NAFLD 23\% of patients had normal values of ALT at the time of biopsy even though fibrosis was observed in 60\% of them\cite{42}. Not surprisingly, the issue of when to perform a liver biopsy in children with suspected NAFLD remains controversial\cite{38,42,47}, and there is no clear standard\cite{47}. Unfortunately, none of the clinical and laboratory predictors of histology appear sufficiently powerful to replace liver biopsy as an accurate noninvasive means of identifying the progression of disease\cite{48}. Nonetheless, there is a considerable, continuous interest in reliable, noninvasive alternatives that will allow the prognosis of pediatric NAFLD to be followed in large community or population-based studies. Recently, transient elastography (TE), using the Fibroscan apparatus, has received increasing attention as a noninvasive means to measure liver stiffness and thus progression in chronic liver disease patients\cite{112}. Accordingly, the study by Nobili \textit{et al.}\cite{113} indicated that TE is an accurate and reproducible methodology to identify, in children and adolescents affected by NASH, those without any degree of fibrosis, or with advanced fibrosis. However, in that study an overlap was observed among patients with lower degrees of liver fibrosis (stages 0 and 1 and stages 1 and 2). Further limitations of that study are related to the acquisition of a highly selected cohort typical of a specialized tertiary care referral center\cite{113}. Thus the conclusions of the study cannot be applied to pediatric populations seen in primary care settings. Nonetheless, the recent results reported in a cohort of pediatric NAFLD indicate that measurement of specific circulating markers of fibrinogenesis or fibrosis through the enhanced liver fibrosis (ELF) test appears to be a promising alternative for discriminating between different stages of fibrosis\cite{114}. Again, further characterization of this test’s performance in larger and less selected cohorts of patients is needed before proposing the use of the ELF panel in clinical practice. Studies in this area are likely to continue.

**CLINICAL PRESENTATION OF NAFLD**

Most children with NAFLD are asymptomatic\cite{20,54}, and elevated levels of aminotransferases are frequently found incidentally or after screening for obesity-related comorbidities\cite{51}. Children may also complain of vague right upper quadrant or epigastric pain, fatigue or malaise. The typical child with NAFLD is an 11-13 year-old, usually male, usually overweight or obese\cite{47}. Some children with NAFLD are tall with large bones and proportionally heavy body weight\cite{92}, consistent with being overnourished.

A thorough history often reveals comorbid conditions related to metabolic syndrome, including hypertension, type 2 diabetes mellitus, dyslipidemia, obstructive sleep apnea, and polycystic ovarian syndrome\cite{39}. In 36\%-49\% of children with NAFLD acanthosis nigricans, a brown to black pigmentation of skin folds and axillae, has been found\cite{41,52}. Acanthosis nigricans may be subtle and can be missed without careful examination\cite{92}. Although acanthosis nigricans may occur in simple childhood obesity, it has been shown to be a cutaneous marker of hyperinsulinemia\cite{103,104}. Keratinocytes have receptors for insulin, and insulin-like growth factors. In hyperinsulinemia, circulating insulin, because of its structural similarity to insulin-like growth factors, binds to these receptors and stimulates cell division, leading to acanthosis\cite{92}.

More than 90\% of children with NAFLD are obese, with central adiposity\cite{41}. On abdominal examination hepatomegaly, with or without splenomegaly, is evident in 33\%-51\% of patients\cite{41,52}. Central adiposity can make organomegaly difficult to identify.

**DIAGNOSIS OF NAFLD**

**Laboratory assessment**

The NASH Clinical Research Network recently failed to identify routine laboratory tests with an adequate discriminating power to replace liver biopsy in evaluating NAFLD pattern and fibrosis severity in children and adolescents\cite{40}. Serum aminotransferases are usually slightly to moderately elevated (less than 1.5 times the upper limit of normal) in NAFLD, but may be higher\cite{114}. The AST: ALT ratio is usually less than one, but this
ratio increases as fibrosis advances\cite{79}. However, aminotransferase levels may remain normal, even with biopsy-proven NASH\cite{117}. In one study evaluating clinical correlation of histopathology in pediatric NAFLD\cite{406}, Patton et al showed that AST was superior to ALT in distinguishing NAFLD patterns and that the addition of ALT to AST did not improve performance. Although these results did not support the use of AST in place of liver biopsy, the strong association found in that study between AST and meaningful histological features in pediatric NAFLD supports current recommendations to use serum aminotransferase levels in screening overweight children\cite{118}. Total and direct bilirubin are typically normal. One laboratory value that may be useful is gamma glutamyl transferase (GGT). In one series of pediatric NAFLD\cite{121}, GGT was elevated in 88% of patients. However, most of them (83.3%) presented with at least one feature of the metabolic syndrome whereas overt metabolic syndrome (i.e. > 3 features) was present in 28.8% children. Recently, elevated serum levels of GGT have been associated with several cardiovascular disease risk factors\cite{119-120,122}. GGT may also act as a marker of the metabolic syndrome\cite{117}. It mediates the uptake of glutathione, an important component of intracellular antioxidant defenses. Although the relationship between the metabolic syndrome and NAFLD in children has not been characterized, GGT may be used as a clinical marker in pediatric NAFLD because its expression is enhanced by oxidative stress and it may be released by conditions inducing cellular stress and insulin resistance\cite{123}, which are key components in the development of NAFLD\cite{122}. Prothrombin time and serum albumin levels are normal, until the development of cirrhosis and liver failure. Thirty per cent of adults with NAFLD have high serum ferritin and 6%-14% have elevated transferrin saturation\cite{109}. These iron indices, however, are not routinely measured in youth, as hemochromatosis is rare in children\cite{109}. Decreased serum adiponectin predicts severity of liver disease in NAFLD, even in the absence of diabetes and obesity, although it remains a research tool and not a diagnostic criterion\cite{97}. Nonspecific autoantibodies, usually against smooth muscle determinants, may be detected in relatively low titers in up to 3% of adults with NAFLD. However, the prevalence in children is unknown\cite{123}.

In diagnosis of NAFLD, a through evaluation and systematic exclusion of other causes of liver disease is necessary; including Wilson’s disease, viral hepatitis, autoimmune hepatitis, alpha-1-antitrypsin deficiency, fatty acid oxidation defects, lipodystrophy, and total parenteral nutrition. Furthermore, drug-induced liver injury (i.e. valproate, methotrexate, tetracycline, amiodarone, prednisone, and synthetic estrogens) should be considered and excluded. Alcohol use, especially in adolescents, must also be excluded.

**Radiological assessment**

Noninvasive imaging techniques, including ultrasound, computed tomography (CT), MRI, and MRS may detect fatty infiltration of the liver but, unlike liver biopsy, they are limited in their ability to detect coexisting inflammation and fibrosis\cite{124}. It has been suggested that unenhanced CT might be useful in the noninvasive quantification of the degree of hepatic steatosis in experimental and in vivo human studies\cite{125,126}. However, a recent study by Pak et al\cite{127} concluded that diagnostic performance of unenhanced CT for quantitative assessment of macrovesicular steatosis is not clinically acceptable. Unenhanced CT does not provide high performance in qualitative diagnosis of macrovesicular steatosis of less than 30%\cite{124}. In addition, CT scanning has the drawback of exposing subjects to ionizing radiation. These two factors limit its potential use in pediatric longitudinal studies\cite{124}. The diagnosis of fatty liver on contrast-enhanced helical CT may also be accurate but is protocol-specific\cite{128}.

**Ultrasound**

Hepatic ultrasound is a relatively inexpensive, noninvasive technique, which is easy to perform and is, therefore, widely used in clinical practice to detect fatty infiltration of the liver. However, sonography is not typically quantitative and a hepatocyte fat content of $\geq 15\%$ to $30\%$ is required to detect ultrasonographic changes\cite{129}. In adults, ultrasound sensitivity has been shown to range from 60% to 94% and specificity from 84% to 95%, respectively\cite{118,133}. In the presence of hepatic fat content of 10% to 19%, ultrasound has a sensitivity of 55%, which rises to 80% in the presence of $>30\%$ fatty infiltration\cite{119}. Furthermore, ultrasound may be technically challenging to perform in patients with significant central obesity. In the presence of morbid obesity\cite{134}, the sensitivity and specificity of ultrasound fall to 49% and 75%, respectively, possibly due to technical problems in performing ultrasound in such patients. Furthermore, ultrasound is operator-dependent\cite{124,134}, and the sonographic evaluation of the liver is based mainly on the subjective visual assessment of hepatic echogenicity and posterior attenuation of the ultrasound beam, with consequent substantial observer variability. In the report by Strauss et al\cite{135}, the mean interobserver and intraobserver agreement rates for the presence of fatty liver were 72% and 76%, respectively. In the same report by Strauss et al\cite{135}, on severity of fatty liver, the initial reading for pairs of observers had 47%-59% interobserver agreement, while the interobserver agreement for the second reading was 59%-64%. The mean agreement rates for pairs of observers were 53% and 62% on the first and second readings. Intraobserver agreement for severity of fatty liver ranged from 55% to 68%. Grading of hepatic fat content using ultrasound has been reported, but it is somewhat subjective and only broad categories of involvement have been reported\cite{130,132,136,137}.

**Magnetic resonance imaging**

MRI, though more costly, is more sensitive than ultrasound in detecting fat and allows for more definitive
hepatic fat quantification when performed using the modified Dixon technique\textsuperscript{[54,138-140]}. Like ultrasound, hepatic MRI involving fast gradient echo does not require conscious sedation in (compliant) children. In fact, the sequence of scan parameters allows simultaneous acquisition of both in-phase and out-of-phase images during the multibreath-hold interval required to cover the entire liver. However, MRI is more appealing than ultrasound to detect minor changes in hepatic fat content associated with steatogenic disorders\textsuperscript{[138]}. While hepatic fibrosis can limit the ability of ultrasound to grade hepatic steatosis, hepatic MRI, based upon chemical shift imaging, is not influenced by the presence of fibrosis in the accurate quantification of the hepatic fat content\textsuperscript{[138]}. In a previous study evaluating hepatic steatosis severity in a series of obese children through both MRI and ultrasound, we found evidence of limitations in ultrasound with regard to grading of steatosis, in comparison with quantitative assessment of HFF by MRI\textsuperscript{[26]}. Ultrasound scores varied across MRI hepatic fat contents. In children in whom ultrasound revealed moderate to severe steatosis, MRI delineated a wide range of hepatic fat content within both categories of ultrasound steatosis severity\textsuperscript{[26]}. This suggests that the utility of ultrasound would appear to be limited by its incapacity to identify fat regression or progression in subjects with NAFLD\textsuperscript{[36]}. The progression of NAFLD in children can be prevented by early weight reduction, which can lessen the degree of fatty infiltration and elicit reversion of the biochemical abnormalities. Thus, a child with NAFLD undergoing a reduction of MRI HFF from 40% to 20% through successful intervention would be unlikely to have a corresponding alteration in ultrasound appearance\textsuperscript{[141]}. However, in our series of children with mild steatosis severity as determined by ultrasound, the pattern of a slight increase in liver echogenicity conflicted with the finding of normal, minimal levels of MRI HFF\textsuperscript{[26]}. Previous investigations have found the slight alterations or accumulation of hepatic fat content as indicated by ultrasound to be equivocal\textsuperscript{[26]}. In contrast, HFF, which is derived from the signal differences between fat and water, gives unequivocal data for the entire spectrum of fatty liver and unlike sonography, is not subject to interpretation or interobserver variation\textsuperscript{[54]}. The clinical efficacy of this technique has been previously demonstrated. Burgert et al showed that obese children with a high HFF were significantly more insulin resistant, compared with those with a low HFF, and had higher triglycerides and lower adiponectin, even after adjustment for BMI-z scores, race/ethnicity, gender, and age\textsuperscript{[26]}. Furthermore, obese children with a high HFF had a significantly greater prevalence of the metabolic syndrome, after controlling for the above confounders\textsuperscript{[26]}. We also previously showed that the increasing severity of MRI fat accumulation were strongly related to fasting hyperinsulinemia and insulin resistance after correction for confounding variables such as SD score-BMI, sex, age and pubertal status\textsuperscript{[26]}. MRS is currently considered being the most accurate for determination of HFF (as a measure of liver triglyceride concentration\textsuperscript{[21]}), especially in patients with less than 10% of fat in the liver\textsuperscript{[21]}. However, MRS demonstrates some limitations in that it is time-consuming, restricted in spatial coverage, and requires off-scan analysis by an expert\textsuperscript{[21]}. Because of these limitations, MRS is not appropriate for widespread use. Furthermore, the main limitation of MRS is that it provides information from small regions of interest in the liver. By contrast, MRI (using the modified Dixon technique) allows evaluation of the presence of fat in the entire liver. To date, however, none of the above imaging modalities allow differentiation of benign steatosis from NASH or have the ability to grade the severity of inflammation\textsuperscript{[36]}.

**CONCLUSION**

Over the last decade, pediatric NAFLD has become the most common form of liver disease in the preadolescent and adolescent age groups. Liver biopsy remains the gold standard and is currently the only way to diagnose NASH. However, the issue of when to perform a liver biopsy in children with suspected NAFLD remains controversial, and there is no clear standard. Thus, it is imperative that reliable, noninvasive means be developed to identify children at greatest risk for progressive disease. Large population-based epidemiologic studies in children are needed to understand the true impact of pediatric NASH on long-term morbidity and mortality.

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